VOLUME 35 JANUARY 1957 NUMBER 1

Canadian Journal of Botany

Editor: D. L. BAILEY

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF BOTANY

(Formerly Section C, Canadian Journal of Research)

Under the authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research, the National Research Council issues THE CANADIAN JOURNAL OF BOTANY and six other journals devoted to the publication, in English or French, of the results of original scientific research. Matters of general policy concerning these journals are the responsibility of a joint Editorial Board consisting of: members representing the National Research Council of Canada; the Editors of the Journals; and members representing the Royal Society of Canada and four other scientific societies.

EDITORIAL BOARD

Representatives of the National Research Council

A. N. CAMPBELL, *University of Manitoba*
G. E. HALL, *University of Western Ontario*
H. G. THODE, *McMaster University*
D. L. THOMSON, *McGill University*
W. H. WATSON (Chairman), *University of Toronto*

Editors of the Journals

D. L. BAILEY, *University of Toronto*
T. W. M. CAMERON, *Macdonald College*
H. E. DUCKWORTH, *McMaster University*
K. A. C. ELLIOTT, *Montreal Neurological Institute*
G. A. LEDINGHAM, *National Research Council*
LÉO MARION, *National Research Council*
R. G. E. MURRAY, *University of Western Ontario*

Representatives of Societies

D. L. BAILEY, *University of Toronto*
Royal Society of Canada
T. W. M. CAMERON, *Macdonald College*
Royal Society of Canada
H. E. DUCKWORTH, *McMaster University*
Royal Society of Canada
Canadian Association of Physicists
K. A. C. ELLIOTT, *Montreal Neurological Institute*
Canadian Physiological Society
R. G. E. MURRAY, *University of Western Ontario*
Canadian Society of Microbiologists
H. G. THODE, *McMaster University*
Chemical Institute of Canada
T. THORVALDSON, *University of Saskatchewan*
Royal Society of Canada

Ex officio

LÉO MARION (Editor-in-Chief), *National Research Council*
F. T. ROSSER, Director, Division of Administration, *National Research Council*

Manuscripts for publication should be submitted to Dr. Léo Marion, Editor-in-Chief, Canadian Journal of Botany, National Research Council, Ottawa 2, Canada.

(For instructions on preparation of copy, see **Notes to Contributors** (inside back cover).)

Proof, correspondence concerning proof, and orders for reprints should be sent to the Manager, Editorial Office (Research Journals), Division of Administration, National Research Council, Ottawa 2, Canada.

Subscriptions, renewals, requests for single or back numbers, and all remittances should be sent to Division of Administration, National Research Council, Ottawa 2, Canada. Remittances should be made payable to the Receiver General of Canada, credit National Research Council.

The journals published, frequency of publication, and prices are:

Canadian Journal of Biochemistry and Physiology	Monthly	$3.00 a year
Canadian Journal of Botany	Bimonthly	$4.00
Canadian Journal of Chemistry	Monthly	$5.00
Canadian Journal of Microbiology	Bimonthly	$3.00
Canadian Journal of Physics	Monthly	$4.00
Canadian Journal of Technology	Bimonthly	$3.00
Canadian Journal of Zoology	Bimonthly	$3.00

The price of single numbers of all journals is 75 cents.

VOLUME 35 1957

Canadian Journal of Botany

Editor: D. L. BAILEY

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

Canadian Journal of Botany

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 35 JANUARY 1957 NUMBER 1

THE BIOSYNTHESIS OF RADIOACTIVE ARABINOSE, XYLOSE, AND GALACTOSE USING DETACHED WHEAT SEEDLINGS[1]

LILLIAN M. COWIE AND G. KROTKOV[2]

Abstract

Five-day-old seedlings of wheat were placed in an atmosphere of $C^{14}O_2$ and permitted to carry on photosynthesis continually for 7 to 8 days. At the end of this time the leaves were extracted with 85% ethanol. The alcohol-insoluble residue was hydrolyzed with 1 *N* hydrochloric acid. The hydrolyzate was deionized, concentrated, and streaked on Whatman No. 3 MM paper. The sugars were separated by one-dimensional chromatography in butanol/pyridine/water and found to be present in the following relative amounts: glucose > xylose > arabinose > galactose.

Introduction

Within the last several years it has been observed that the breakdown of glucose to pyruvate may occur not only via the Embden–Meyerhof scheme, but also according to the phosphogluconate shunt (1, 2, 3, 5, 6). The appearance of ribose and ribulose (9) during the operation of such a shunt indicates the possible manner of formation of these two pentoses and raises the question of the origin of xylose and arabinose, which are known to be widely found in plants (8). It was thought that the availability of radioactive arabinose and xylose would greatly facilitate subsequent investigations of this question, and experiments described below were carried out with the objective of obtaining such sugars.

In principle, the technique proposed for such a synthesis calls for permitting some green plants to carry on photosynthesis in an atmosphere containing $C^{14}O_2$ and then isolating the radioactive sugars. Since pentoses are present mainly as constituents of polysaccharides in the cell walls, it would be advisable to allow the plants actually to grow during the period of photosynthesis. A chromatographic survey of sugars in various plant families revealed that wheat leaves are rich in pentosans (4), and wheat seedlings have been used, therefore, as the experimental plants.

[1]Manuscript received August 8, 1956.
Contribution from the Department of Biology, Queen's University, Kingston, Ontario. With financial help from the Research Council of Ontario and the Committee on Scientific Research of Queen's University.

[2]Department of Biology, Queen's University.

[The previous number of Can. J. Botany (**34**, 825–991) was issued November 30, 1956.]

Method

Seeds* of Marquis wheat were spread on Vermiculite, covered with damp paper towels, and left at 21° C. Germination proceeded at once. The towels were removed after 3 days and from this time on the seedlings were in continuous light. At the end of 5 days those seedlings were used the shoots of which were 5 to 6 cm. in length. At this stage the primary leaf either had broken through the coleoptile or would do so in a few hours' time. The remaining part of the seed, the endosperm and seed coat, and the larger part of the roots, were cut off, care being taken to leave the basal leaf meristem intact. Leaves were inserted into 10-ml. beakers, 10 plants to each beaker, and supplied with 0.1 strength Hoagland solution. The beakers were placed in an 8-liter desiccator closed with a rubber stopper provided with inlet and outlet glass tubes for the evacuation of the air around the seedlings. A 4-ft. length of coiled copper tubing 3/16 in. in diameter was inserted under the lid of the desiccator. Cold water was circulated through this coil to remove excess heat during the experiment. In the bottom of the desiccator was placed about 100 ml. of water containing sulphuric acid at a concentration of 1%. The water maintained humidity within the chamber. It was acidified in order to reduce solution of the radioactive carbon dioxide in it and the waste of carbon-14 which would result.

The desiccator was evacuated to a pressure of about 20 cm. of mercury and then filled in the usual manner (13) with 5% carbon dioxide, containing 0.5 to 1 mc. of carbon-14. Then the pressure inside was brought to normal and the desiccator was illuminated with eight 200-watt incandescent bulbs, the light of which passed through rectangular glass museum jars, filled with water to remove part of the heat.

After 3 days of continuous photosynthesis, the desiccator was aerated with carbon-dioxide-free air and the unassimilated carbon dioxide was absorbed in sodium hydroxide and determined by titration. A new charge of C^{14}-carbon dioxide was given to the seedlings and they were permitted to carry on photosynthesis for two additional periods of illumination. More nutrient solution was supplied to the seedlings after the second period of illumination.

During all three periods the plants remained turgid, retained a good color, and continued to elongate. An estimate of the growth made at the end of the final period showed that the plants in each of the experiments carried out had increased in length and in fresh weight from two to three times. Two experiments were carried out; a summary of them is given in Table I.

At the end of each experiment, the plants were extracted for 8 hours in a Soxhlet extractor with 85% ethanol. The alcohol-insoluble residue was hydrolyzed under a reflux condenser with 1 *N* hydrochloric acid in a boiling water bath for 1½ hours. The hydrolyzate was filtered and diluted with four times its volume of water. It was then deionized by passage through

*The authors wish to express their thanks to Dr. J. G. C. Fraser of the Central Experimental Farm, Ottawa, who kindly provided these seeds.

PLATE I

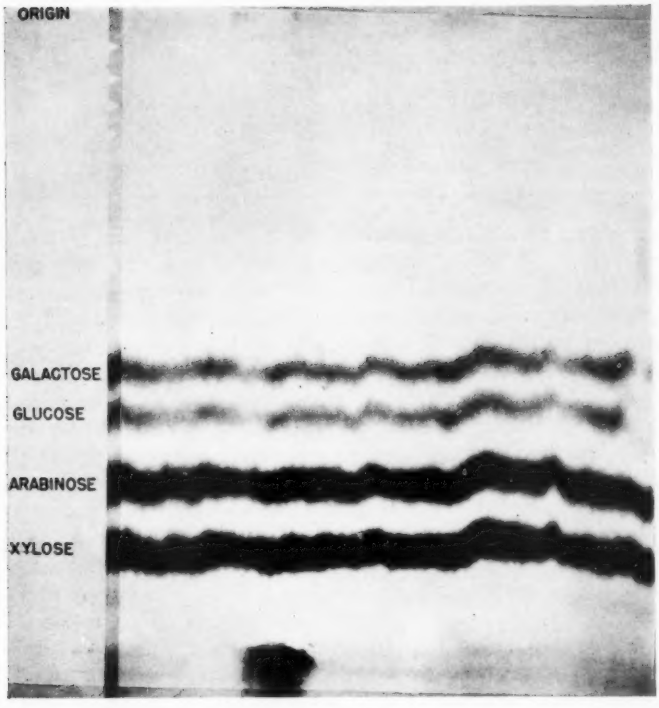


FIG. 1. Autoradiograph of deionized acid hydrolyzate of alcohol-insoluble residue from wheat seedlings.

TABLE I

SUMMARY OF PHOTOSYNTHETIC CONDITIONS

Expt. No.	No. of plants	Period No.	Duration, days	Carbon dioxide: Offered Mg.	Carbon dioxide: Offered Mc.	Conc. in chamber, %	Amount assim'd as % of amount offered	Total activity assim'd, mc.
1	100	1	3	745	0.5	5	100	
		2	2	745	0.5	5	100	
		3	3	745	0.5	5	100	1.5
2	50	1	2	800	1.0	5.5	73	
		2	2	800	1.0	5.5	100	
		3	3	800	1.0	5.5	85	2.6

Amberlite 120-H and Duolite C-3 ion exchange resins. The effluent was clear and colorless. It was concentrated *in vacuo* at 30° C. to about 5 ml.

Tnis concentrate was streaked on 18 in. × 22 in. sheets of Whatman No. 3 MM paper. To ensure good separation of the glucose and galactose present it is usually advisable to apply the mixture in such a way that the amount of glucose per linear inch is little more than 200 μg. These were chromatogrammed using butanol/pyridine/water in the proportion 3/1/1.

The concentrate was found to contain glucose, galactose, xylose, and arabinose. Using the adopted technique, separation of the pentoses presented no difficulty, while glucose and galactose ran very close. By interrupting the flow of solvent, it was possible, however, to obtain a better resolution (10). The solvent was allowed to flow for periods of 12, 14, and 16 hours, the sheets being dried between runs for ½ hour at room temperature. Bands of the various sugars were located by radioautography and one of the radioautographs obtained is shown in Fig. 1.

The bands of various sugars were eluted and made to volume. The amounts of the hexoses in them were determined using the Morris anthrone method (12), while Drury's adaptation (7) of Mejbaum' orcinol method (11) was used for determining the pentoses. Aliquots of these concentrates were also plated and counted in a methane flow counter. Table II gives the specific activities and the amounts of various sugars isolated in both experiments.

TABLE II

SPECIFIC ACTIVITIES AND TOTAL AMOUNTS OF RADIOACTIVE SUGARS OBTAINED FROM THE ACID-HYDROLYZABLE FRACTION OF YOUNG WHEAT PLANTS

Sugar	Experiment 1: Specific activity, μc. per mg.	Experiment 1: Total amount, mg.	Experiment 2: Specific activity, μc. per mg.	Experiment 2: Total amount, mg.
Glucose	0.28	157	2.11	125
Galactose	0.26	37	1.82	19
Arabinose	0.31	62	1.75	46
Xylose	0.35	124	1.78	75

TABLE III

SPECIFIC ACTIVITIES AND TOTAL AMOUNTS OF RADIOACTIVE SUGARS OBTAINED FROM THE ALCOHOL-SOLUBLE FRACTION OF YOUNG WHEAT PLANTS

	Experiment 1		Experiment 2	
Sugar	Specific activity, μc. per mg.	Total amount, mg.	Specific activity, μc. per mg.	Total amount, mg.
Sucrose	0.88	50	0.99	0.90
Glucose	0.44	67	0.47	0.27
Fructose	0.41	38	2.58	0.19

In addition to the four sugars just listed above, it is possible to recover variable amounts of sucrose, glucose, and fructose from the alcohol soluble extract of the radioactive seedlings. Their amounts, however, are relatively small, as is seen from Table III. The use of wheat seedlings, therefore, for the isolation of radioactive sugars from the alcohol soluble extract is not a satisfactory one. Were it desirable to biosynthesize such sugars, one should use some other plants, for example tobacco, as described by Vittorio (13, 14). The residue after the extraction of the hexoses and pentoses yields radioactive cellulose.

References

1. AXELROD, B., BANDURSKI, R. S., GREINER, C. M., and JANG, R. The metabolism of hexose and pentose phosphates in higher plants. J. Biol. Chem. **202,** 619–634 (1953).
2. BARNETT, R. C., STAFFORD, H. A., CONN, E. E., and VENNESLAND, B. Phosphogluconic dehydrogenase in higher plants. Plant Phys. J. **28,** 115–122 (1952).
3. BEEVERS, H. and GIBBS, M. The direct oxidation pathway in plant respiration. Plant Physiol. **29,** 322–324 (1954).
4. BIDWELL, R. G. S., KROTKOV, G., and REED, G. B. Paper chromatography of sugars in plants. Can. J. Botany, **30,** 291–305 (1952).
5. DICKENS, F. Oxidation of phosphohexonate and pentose phosphoric acids by yeast enzymes. Biochem. J. **32,** 1626–1644 (1938).
6. DICKENS, F. and GLOCK, G. E. Direct oxidation of glucose-6-phosphate, 6-phosphogluconate and pentose-5-phosphates by enzymes of animal origin. Biochem. J. **50,** 81–95 (1951).
7. DRURY, H. F. Identification and estimation of pentoses in the presence of glucose. Arch. Biochem. **19,** 455–466 (1948).
8. HIRST, E. L. The occurrence and significance of the pentose sugars in nature, and their relation to the hexoses. J. Chem. Soc. Pt. I, 522–533 (1949).
9. HORECKER, B. L., SMYRNIOTIS, P. Z., and SEEGMILLER, J. E. The enzymatic conversion of 6-phosphogluconate to ribulose-5-phosphate and ribose-5-phosphate. J. Biol. Chem. **193,** 383–396 (1951).
10. JEANES, A., WISE, C. S., and DIMMLER, R. J. Improved techniques in paper chromatography of carbohydrates. Anal. Chem. **23,** 415–420 (1951).
11. MEJBAUM, W. Über die Bestimmung kleiner Pentosemengen insbesondere in Derivatives der Adenylsaüre. Z. physiol. Chem. **258,** 117–120 (1939).
12. MORRIS, D. I. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science, **107,** 254–255 (1948).
13. VITTORIO, P. V. The use of carbon-14 in the study of starch and sugar synthesis in detached tobacco leaves. Ph.D. Thesis, Queen's University, Kingston, Ontario (1953).
14. VITTORIO, P. V., KROTKOV, G., and REED, G. B. Synthesis of radioactive sucrose by tobacco leaves from C^{14} uniformly labelled glucose and glucose-1-phosphate. Can. J. Botany, **32,** 369–377 (1954).

THE CONSTITUTION OF SUGAR BEET SEEDLINGS ASSOCIATED WITH CHEMICALLY INDUCED IMPROVEMENT IN THEIR LOW TEMPERATURE RESISTANCE[1]

SHERWOOD R. MILLER[2] AND WILLIAM G. CORNS

Abstract

The work concerned some chemical and physiological characteristics of sugar beet seedlings grown in darkness in vermiculite moistened with water or with 4, 8, 12 p.p.m. solutions of TCA (sodium salt of trichloroacetic acid) or of Dalapon (sodium salt of 2,2-dichloropropionic acid). The chemical treatments, which were effective in improving the cold resistance of the seedling shoots, decreased their moisture content, increased their rate of water loss, and decreased their resistance to desiccation; increased the sugar and the dry matter content; and had no appreciable effect on the amount of total nitrogen or of water soluble nitrogen in the seedlings.

Introduction

Earlier work has shown that Dalapon (2,2-dichloropropionic acid) and TCA (trichloroacetic acid) improve the low temperature resistance of sugar beet seedlings treated under appropriate laboratory conditions (2, 3). The present paper is based on results from part of a field and laboratory investigation by the senior author (7) during which an attempt was made to clarify differences between chemically treated and untreated sugar beets. The current report is confined to some experiments with etiolated seedling shoots prepared and analyzed under suitably controlled conditions.

Materials and Methods

In preparation for the various tests the beet seedling material, variety Kuhn, was grown in vermiculite in the manner previously outlined (2). The chosen concentrations of 4, 8, and 12 p.p.m. Dalapon or TCA were intended to involve a range of plant tolerance without causing objectionable stunting by the chemicals. Measurements of shoot and root lengths in typical experiments showed that the 12 p.p.m. concentration of either chemical could cause some inhibition of growth but the treatments with 4 and 8 p.p.m. were without such effect. All three rates were effective in increasing the low temperature resistance of the seedlings by amounts comparable to those reported earlier in similar experiments with Dalapon and TCA (3).

In order to study rates of loss in weight, quadruplicate samples of 25 freshly detached shoots from each treatment were desiccated at room temperature and their weights recorded at intervals over a 24 hour period. In supplementary tests the stem pieces from different treatments were placed in water for several hours after various periods of desiccation. The numbers and percentages of apparently undamaged survivors were then recorded.

[1]Manuscript received August 22, 1956.
Contribution from the Department of Plant Science, University of Alberta, Edmonton, Alberta.
[2]Present address: Central Experimental Farm, Ottawa.

Total moisture and dry matter percentages were determined on quadruplicate samples of approximately 100 seedlings which were weighed fresh and dried at 100° C. to constant weight.

The total nitrogen of the seedlings was determined on the same samples used for finding moisture percentage. The dried samples were ground in a mortar and then redried at 100° C. to constant weight. Duplicate aliquots of 10 to 35 mg. material were weighed on cigarette paper and transferred to micro-Kjeldahl flasks for digestion in accordance with the official micro-Kjeldahl method (1).

For analysis of water soluble nitrogen, duplicate 50 mg. samples of dry material were weighed into 50 ml. Erlenmeyer flasks containing 20 ml. water. The flasks and contents were then shaken for 16 hours to extract the water soluble fractions from the samples. After filtration of this extract, an aliquot of each filtrate was digested in the micro-Kjeldahl flask with the aid of superoxol and the Galston and Dalberg mixture (4). The method noted for total nitrogen was used to complete the procedure.

In determinations of reducing sugar and total sugar, 50 seedlings from each treatment were weighed into duplicate Soxhlet thimbles for 6 hours' extraction with 80% ethanol. Following extraction, the alcohol was driven off and the extract cleared with saturated solutions of neutral lead acetate and of disodium phosphate. After filtration through a Buchner funnel, reducing sugars were determined by the method of Hassid (5, 6). An aliquot of the cleared extract was hydrolyzed with hydrochloric acid and after 24 hours neutralized with sodium carbonate preparatory to the determination of total sugars by the same method used for reducing sugars. Results were expressed as % glucose based on dry weight.

Results

Data showing differences in rates of drying of the seedling shoots from different treatments appear in Table I.

When trials were carried out to ascertain the numbers of apparently undamaged shoots remaining after periods of desiccation ranging from 6 to 10 hours, followed by placement of this material in water overnight at room temperature, the results were in line with the trends shown above in Table I. Untreated material clearly had the higher potential for survival, thus exhibiting an inverse correlation between weight loss, which presumably was mainly water loss, and survival. No attempt was made to determine optimum temperatures and durations of desiccation associated with potential ranges of differences within and between treatments. The following examples in Table II will, however, illustrate the results that were obtained.

Table III summarizes the results from the various other determinations made on sugar beet seedlings according to methods outlined earlier.

These results show that moisture content of chemically treated seedlings declined with increasing concentration of chemicals used. Conversely there was a parallel increase in dry matter. Associated with the increase

TABLE I

PERCENTAGE OF FRESH WEIGHT LOST FROM SUGAR BEET SEEDLING SHOOTS AFTER VARIOUS PERIODS OF DESICCATION AT ROOM TEMPERATURE

	Desiccation period in hours			
	1	2	5	24
Dalapon treatment				
0 (check)	16.80	29.66	60.09	85.11
4 p.p.m.	17.79	33.46*	66.34*	88.37*
8 p.p.m.	19.27**	34.77**	69.58**	89.68**
12 p.p.m.	20.41**	36.08**	71.58**	90.17**
L.S.D. at 5% level	1.59	3.01	6.01	3.10
L.S.D. at 1% level	2.29	4.32	8.64	4.46
TCA treatment				
0	15.44	28.00	59.40	92.31
4 p.p.m.	18.80*	30.34	68.83**	94.32**
8 p.p.m.	17.25	31.42*	70.34**	94.42**
12 p.p.m.	19.90**	36.86**	76.65**	94.26**
L.S.D. at 5% level	2.75	3.27	4.06	0.96
L.S.D. at 1% level	3.95	4.70	5.83	1.39

*Significantly different from check at 5% level.
**Significantly different from check at 1% level.

TABLE II

PERCENTAGES OF SURVIVING SUGAR BEET SEEDLING STEMS FOLLOWING DESICCATION AND WATER IMMERSION

		Chemical concentration, p.p.m.				
		0	2	4	8	12
Dalapon treatment						
10 hours' desiccation	(*a*)	48	—	20	15	7
	(*b*)	66	—	27	21	14
TCA treatment						
7 hours' desiccation	(*a*)	98	90	76	59	—
	(*b*)	94	85	76	28	—

in dry matter was a progressive increase in both reducing sugar and total sugar. Reducing sugar made up the bulk of the total sugar. With respect to the differences between amounts of total sugar and corresponding reducing sugar, there was a consistent trend for decline in non-reducing sugar with increasing concentration of Dalapon treatment, but not with TCA. Moreover there was a consistent increase in grams of sugar per gram of dry matter accompanying the increasing concentrations of treatments with both chemicals. There was apparently no appreciable change in amount of total nitrogen, or in water soluble nitrogen regardless of treatment used.

In summary it appears reasonable to conclude that the combination of factors, viz.: organization permitting increased rate of water loss, decreased total moisture content, and increased sugar content resulting in increased

TABLE III

COMPOSITION OF SUGAR BEET SEEDLINGS FOLLOWING CHEMICAL TREATMENTS KNOWN TO IMPROVE THEIR RESISTANCE TO LOW TEMPERATURE

(Results in percentage, except final column)

	Moisture	Dry matter	Reducing sugar	Total sugar	Nitrogen	Water soluble nitrogen	G. sugar per g. dry matter
Dalapon treatment							
0 (check)	95.19	4.81	12.66	13.93	3.32	0.54	0.67
4 p.p.m.	94.97	5.03	14.31	15.31	3.30	0.58	0.77
8 p.p.m.	94.67	5.33	16.14	17.07	3.28	0.57	0.91
12 p.p.m.	94.63	5.37	17.13	17.69	3.36	0.58	0.95
TCA treatment							
0	95.16	4.84	11.78	11.98	3.21	0.60	0.58
4 p.p.m.	94.91	5.09	13.95	14.73	3.02	0.57	0.75
8 p.p.m.	94.78	5.22	13.79	13.79	3.34	0.51	0.72
12 p.p.m.	94.57	5.43	15.28	15.65	3.28	0.57	0.85

solution concentration has direct bearing on the superior resistance to low temperature exhibited by seedlings treated with Dalapon and TCA. Of these factors, the one of most questionable significance might be the increased rate of water loss, in view of the very short time available for this process to express itself during the rapid freezing technique employed for evaluation of treatments. Investigation of the relationship of the above findings to green plant composition and frost resistance under natural conditions offers interesting basic and practical problems for further research.

Acknowledgment

We are grateful for the interest in, and financial support of, this work by the Federal Department of Agriculture, the National Research Council, and the University of Alberta, and for technical assistance by R. Schraa. Dow Chemical Co. and Canadian Sugar Factories Ltd. kindly donated chemicals and sugar beet seed respectively.

References

1. ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. Official and tentative methods of analysis of the A.O.A.C. 6th ed. Washington, D.C. 1945.
2. CORNS, W. G. Effects of various chemical and cold-hardening treatments on resistance of sugar beet seedlings to freezing. Can. J. Botany, **34**, 154–158 (1956).
3. CORNS, W. G. and SCHWERDTFEGER, G. Improvement in low temperature resistance of sugar beet and garden beet seedlings treated with sodium TCA and Dalapon. Can. J. Agr. Sci. **34**, 639–641 (1954).
4. GALSTON, A. W. and DALBERG, L. Y. The adaptive formation and physiological significance of indoleacetic acid oxidase. Am. J. Botany, **41**, 373–380 (1954).
5. HASSID, W. Z. Determination of reducing sugars and sucrose in plant materials. Ind. Eng. Chem. Anal. Ed. **8**, 138–140 (1936).
6. HASSID, W. Z. Determination of sugars in plants by oxidation with ferricyanide and ceric sulfate titration. Ind. Eng. Chem. Anal. Ed. **9**, 228–229 (1937).
7. MILLER, S. R. Some effects of growth substances on low temperature resistance and quality of sugar beets. M. Sc. Thesis, Department of Plant Science, University of Alberta, June, 1956.

SOLUBLE CARBOHYDRATES OF WHEAT STEMS AND LEAVES[1]

L. E. LOPATECKI,[2] E. L. LONGAIR,[2] AND C. W. FARSTAD[3]

Abstract

Chromatographic evidence indicated that glucose, fructose, and sucrose were present in wheat stems and leaves at stages of growth from early shot blade to maturity. A number of non-reducing oligosaccharides soluble in 80% ethanol were also present in both leaves and stems during this period. Simultaneously, at least one water-soluble oligosaccharide which is insoluble in 80% ethanol appeared in the stems. All oligosaccharides examined produced only glucose and fructose upon complete hydrolysis with invertase or acid.

Introduction

Practical resistance against the wheat stem sawfly, *Cephus cinctus* Nort., is known to be associated with the pith content of the wheat stem (9). Farstad (4) has suggested a lack of energy-containing foods in the pith of solid stem varieties, as a factor contributing to this resistance.

To test the above hypothesis, a survey of the available food materials present in wheat stems at successive stages of growth was begun. The present paper submits chromatographic data on the water-soluble carbohydrates of stems and makes a comparison with similar carbohydrates present in the leaves.

Little information was found on the water-soluble carbohydrates of wheat stems. Bradfield and Flood (1) reported that just prior to yellowing, stalks of Square Head wheat, including leaf blades and sheaths, produced chromatograms indicating fructose, glucose, sucrose, and sorbitol. Additional unresolved chromatographic spots suggested the presence of five or more carbohydrates of higher molecular weights. The distribution of carbohydrates between stems and leaves was not determined. It has been shown by Roberts (11) and confirmed by Ward (12) that sucrose is the only soluble carbohydrate of any consequence in wheat leaves at early stages of growth.

Materials and Methods

Four wheats were selected for analysis: three varieties of *Triticum vulgare* Vill., Thatcher (hollow stem and susceptible), Rescue (solid stem and resistant), and S-615; and one variety of *T. durum* Desf., Golden Ball (solid stem and resistant).

Experimental procedures were based largely upon the methods of Laidlaw and Reid (7) for estimating free sugars and fructosans of grasses. An outline of this experimental procedure is given below.

[1]Manuscript received July 10, 1956.
Contribution No. 323, Chemistry Division, and No. 3450, Entomology Division, Science Service, Department of Agriculture, Ottawa, Canada.
[2]Chemistry Section, Science Service Laboratory, Lethbridge, Alberta.
[3]Field Crop Insect Section, Science Service Laboratory, Lethbridge, Alberta.

Preparation of Plant Samples

Randomized plots of the four wheat varieties were grown both on dry land and on land which had at some time been irrigated. No difference was found in the samples taken from these two sources.

Samples of stems were selected which would approximate as closely as possible that portion of stem utilized as food by the sawfly. These were collected at intervals from the stage of early shot blade until the wheat was almost ripe. This period of sampling corresponded with the time from hatching of the egg until the larva approached maturity within the stem. Samples were taken between 9.00 and 10.00 a.m. on clear sunlight mornings. Stems were cut at ground level, and the head was removed at the first node along with all outer leaf coverings; leaves from the variety Thatcher only were retained for analysis. As rapidly as possible after collection, stems and leaves were immersed in boiling 87% ethanol and boiled for 3 minutes. Prior to further processing, these samples were stored at −5° C.

Preparation of Plant Extracts

Following decantation of alcohol, the plant material was treated in a Waring blendor for 3 minutes. The plant debris was then transferred to a Soxhlet and extracted with 80% ethanol for 3 hours. Chromatographic examinations of the latter extracts showed that extraction of alcohol-soluble carbohydrates was complete.

The alcoholic extracts were then evaporated to a sirup under vacuum at 39° C., and the residue taken up in water. Clarification was accomplished with equivalent quantities of barium hydroxide and aluminum sulphate, as described by Browne and Zerban (2), followed by deionization through amberlite IRC–50 and IR–4B. Specific resistance of deionized solutions was kept above 35,000 ohms to obtain chromatographic spots without tails.

The Soxhlet residues were re-extracted with boiling water to remove water-soluble fructosans. This fructosan extract was then clarified, deionized, and chromatographed in the usual way. Precipitation of the major component from a portion of the extract was accomplished in 75% ethanol. The precipitated fructosan was then dissolved in water and again chromatographed, both before and after hydrolysis.

Identification of Carbohydrates

(*a*) *Chromatography*

Chromatographic separation of carbohydrates was accomplished by the paper partition procedure of Partridge (8), using butanol, acetic acid, water solvent (4:1:5), and Whatman No. 4 paper. As control sugars, spots of 1.0% solutions of glucose, fructose, and sucrose were run on each paper. Development time varied from 16 hours for hexose separation to 12 days for that of oligosaccharides.

Chromatograms were sprayed with a 14:1 mixture of the naphthoresorcinol–trichloroacetic acid reagent of Partridge (8) and the benzidine reagent of Horrocks (5). This mixture was equally sensitive to aldo and keto sugars. After the sprayed chromatograms were heated for 3 minutes at 120° C.,

fructose appeared bright yellow, glucose dark brown, while sucrose and oligosaccharides were of intermediate color. Reducing activity was examined with the silver nitrate – ammonia spray of Partridge (8).

When carbohydrates were eluted from the developed chromatogram, the carbohydrate zones were estimated from strips removed previously from the edge of the paper. Elution was carried out at room temperature by the method of Laidlaw and Reid (6). The milligram quantities of carbohydrate required for hydrolytic and specific color tests were obtained by running separations on Whatman 3MM paper.

(*b*) *Hydrolyses*

Acid and enzyme hydrolyses were carried out on the sucrose and oligosaccharide fractions eluted from chromatograms, and on the purified fructosan. Acid hydrolysis was performed by heating the carbohydrates at 100° C. for periods of 2.5, 5, and 10 minutes in 0.125% sulphuric acid. Tests for hydrolysis with invertase and emulsin were performed by adding 0.1% of commercial enzyme preparation to the carbohydrates and incubating the mixture for 4 hours at room temperature. The sugars formed were then identified chromatographically.

(*c*) *Specific Tests*

The Raybin reaction with diazouracil was performed on sucrose isolated chromatographically. This reaction is reported to be specific for compounds containing the fructofuranose–glucose linkage of sucrose (10).

A confirmatory test for fructose was done by treating the material with cobaltous chloride and ammonia (3).

Results and Discussion

Fig. 1 illustrates chromatograms produced from alcoholic extracts of stems and leaves of Thatcher. R_F values suggested that the three sugars present at early and late shot blade were fructose, glucose, and sucrose. Further confirmation of the identity of these sugars was given by color reactions. Thus all three sugars produced characteristic colors when sprayed with naphthoresorcinol–benzidine mixture. Fructose, which was eluted from chromatograms, produced a violet with cobaltous chloride and ammonia, while sucrose gave a green with diazouracil, which turned blue when magnesium was added.

FIG. 1 shows also that following shot blade, a series of oligosaccharides with lower R_F values appeared in both stem and leaf extracts. Prolonged chromatographic development of the stem extract separated the oligosaccharides into at least six separate entities. The two most mobile components of this fraction possessed R_F values very close to that of the trisaccharide raffinose. On the other hand, solubility of the slowest moving members in 80% alcohol probably limits the upper molecular size to 10 hexose units.

The components of the oligosaccharide fraction gave a negative ammoniacal silver nitrate reaction, were hydrolyzed completely within 5 minutes

in the presence of 0.125% sulphuric acid, and were hydrolyzed completely by invertase but not by emulsin. Fructose and glucose were the only sugars produced by hydrolysis.

Fig. 1 shows additionally that stems and leaves of Thatcher possessed a similar distribution of soluble carbohydrates. Thus fructose, glucose, and sucrose were present in both stems and leaves from early shot blade until maturity. Fructose and glucose in the stem remained fairly constant throughout all periods of growth, while in the leaves these two sugars tended to accumulate to a maximum around the milk stage. The accumulation of sucrose during the milk and dough stage was noticeable in both stems and leaves. Oligosaccharides, on the other hand, appeared slightly earlier in the leaves, but were present in quantity in leaves and stems only during the milk and dough stage. When the water-soluble fructosan fraction from Thatcher stems was chromatographed, one major, slow-moving component appeared, together with traces of oligosaccharides. Following repeated precipitation from 75% ethanol, the major component appeared homogenous when re-chromatographed. This component was non-reducing, and was rapidly hydrolyzed by invertase and acid but not by emulsin. Fructose was the main product of hydrolysis, accompanied by a much lower concentration of glucose.

The qualitative distribution of soluble carbohydrates for the varieties Golden Ball, Rescue, and S–615 was found similar to that of Thatcher. However, the quantities present, as judged by density of chromatographic spots, were noticeably different at some stages of growth. Investigations are proceeding upon the quantitative accumulation of these carbohydrates and upon the final identification of the compounds involved.

References

1. Bradfield, A. E. and Flood, A. E. Soluble carbohydrates of fruit plants. Nature, **166**, 264–265 (1950).
2. Browne, C. A. and Zerban, F. W. Physical and chemical methods of sugar analysis. 3rd ed. John Wiley & Sons, Inc., New York. 1948.
3. Dehn, W. M., Jackson, K. E., and Ballard, D. A. Identification of common carbohydrates. Ind. Eng. Chem. Anal. Ed. **4**, 413–415 (1932).
4. Farstad, C. W. A study of the development of western wheat stem sawfly, *Cephus cinctus* Nort., in various host plants as an index of resistance. Abstract, Doctor's Thesis, Iowa State College. 1940.
5. Horrocks, R. H. Paper partition chromatography of reducing sugars with benzidine as a spraying reagent. Nature, **164**, 444 (1949).
6. Laidlaw, R. A. and Reid, S. G. Filter-paper chromatography: Extraction of the sugars from the paper at room temperature. Nature, **166**, 476–477 (1950).
7. Laidlaw, R. A. and Reid, S. C. Analytical studies on the carbohydrates of grasses and clovers. I. Development of methods for the estimation of the free sugars and fructosan content. J. Sci. Food Agr. **3**, 19–25 (1952).
8. Partridge, S. M., with a note by Westall, R. G. Filter-paper partition chromatography of sugars. Biochem. J. **42**, 238–250 (1948).
9. Platt, A. W. and Farstad, C. W. The reaction of wheat varieties to wheat stem sawfly attack. Sci. Agr. **26**, 231–247 (1946).
10. Raybin, H. The direct demonstration of the sucrose linkage in the oligosaccharides. J. Am. Chem. Soc. **59**, 1402–1403 (1937).
11. Roberts, D. W. A. Qualitative and quantitative determination of wheat leaf carbohydrates. Can. J. Research, C, **28**, 754–779 (1950).
12. Ward, G. M. The isolation and identification of sucrose from the seedling wheat leaf. Can. J. Botany, **31**, 75–80 (1953).

PLATE I

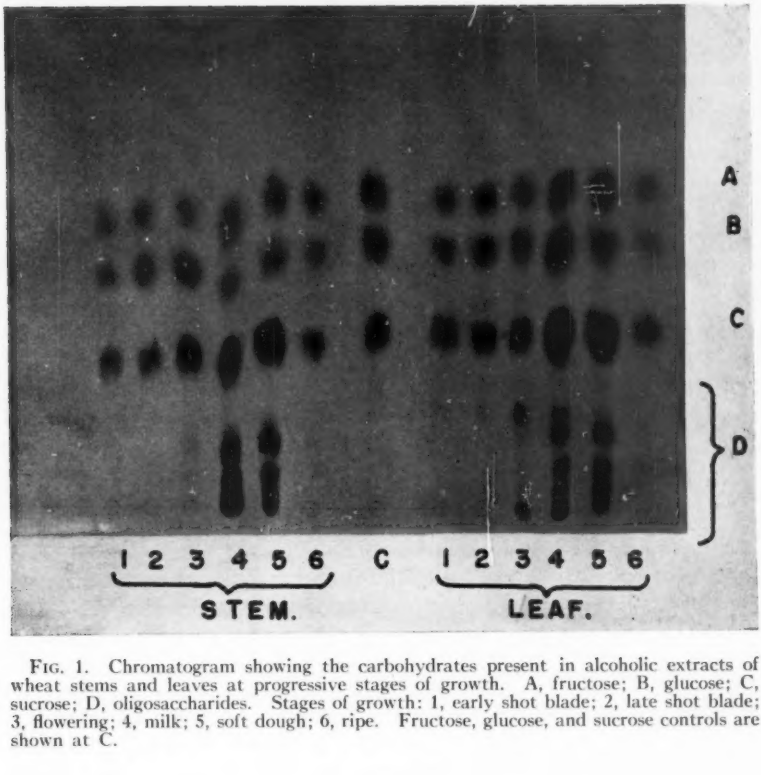


FIG. 1. Chromatogram showing the carbohydrates present in alcoholic extracts of wheat stems and leaves at progressive stages of growth. A, fructose; B, glucose; C, sucrose; D, oligosaccharides. Stages of growth: 1, early shot blade; 2, late shot blade; 3, flowering; 4, milk; 5, soft dough; 6, ripe. Fructose, glucose, and sucrose controls are shown at C.

DEVELOPMENTAL STUDIES OF ELODEA CANADENSIS MICHX.

I. MORPHOLOGICAL DEVELOPMENT AT THE SHOOT APEX[1]

HUGH M. DALE[2]

Abstract

The shoot apex consists of a few initial cells at the tip of a thimble devoid of leaf initials for at least 100 μ. Leaf primorida are initiated from the superficial layer of cells, whereas branch buds arising among the very youngest leaf primordia are produced deeper in the apex. Chinks occur where three or more cell walls come together. The tissue of the stem for the first 200 μ has no internodes. Two squamulae intervaginales lie on the adaxial surface of each leaf with which their development is associated. Internodes are initiated by the longitudinal growth and division of cells from the bottom of the leaf insertion disks. Cells of the young node divide longitudinally to increase the diameter of the nodal disk and to split the intercalary meristem into segments. Internodes are thus initiated with lacunae. Cells destined to become wood vacuolate at the seventh leaf whorl. Scalariform thickenings are produced but quickly disintegrate along with the rest of the xylem cells leaving a lacuna in the center of the stem. The bast surrounding the central xylem differentiates only slightly, beginning at the 20th leaf whorl, whereas the leaf traces and vertical cortical strands are apparent in younger tissue.

Introduction

The interest in *Elodea* which initiated these studies stems from the consideration of the development of air spaces in vascular plants. Intercellular spaces are particularly a feature of parenchymous tissues where they occur as chinks at the junction of three or more cell walls or as larger more elaborate ones such as those in spongy mesophyll of leaves and those in the tissues of aquatic plants. The larger intercellular spaces at maturity contain gases whose composition varies with time. The origin and initial development of intercellular spaces appear to be similar; however their later development follows one of two courses. Either they are the result of death and disintegration of cells, or they result from the splitting apart of cell walls and the later division of cells surrounding the space in such a way that it enlarges into a lacuna. The serious lack of work on these latter, the schizogenetic air spaces, has been pointed out by Sifton (16).

Elodea has proved a good subject for this study, as its basic structure is simple, consisting of a long segmented stem of nodes and internodes produced from an apical meristem that overwinters in the water. The roots, branches, flowers, and leaves are all nodal outgrowths, while the internodes contain a double ring of large lacunae and numerous smaller ones. Branches form new apical growing points and when senility and decay follow to the point of branching, two separate sections are formed. The plant spreads chiefly by this method. This asexual reproduction is a great advantage to an experimentor, since it eliminates the necessity of developing a genetically pure

[1]Manuscript received August 8, 1956.
Contribution from the Department of Botany, University of Toronto, Toronto, Ontario. Part of a thesis presented at the University of Toronto in partial fulfilment of the requirements of the degree of Doctor of Philosophy.
[2]Department of Botany, University of Toronto.

stock. Instead, it is possible to produce thousands of apices from a single individual and carry out experiments upon discrete parts of the same plant. Further, the flowers are axillary so that the apex of the main shoot does not develop from a vegetative into a flowering type. The material for the work presented here came from one small clump in Grenadier Pond in High Park, Toronto, and was grown and propagated in aquaria in the greenhouses of the Department of Botany, University of Toronto.

The Shoot Apex

Figs. 1 and 2 are photomicrographs representing median sections, 5 microns thick, of the shoot apex of *Elodea canadensis*. The two figures are of adjacent sections in a series. Owing to a slight bend in the axis no one section was median throughout its length. This apex, although grown in the laboratory, exhibits developmental stages identical with those found when the plant is grown in its natural habitat, with the difference that the development is slightly retarded. At the top of Fig. 1 are the meristematic initial cells. All other cells are the result of their division or the division of their progeny. Almost all the cells in this plate, Figs. 1 and 2, are meristematic. The notable exception is the central row of enlarged cells extending from the bottom of Fig. 1 through Fig. 2. These cells will be discussed later.

1. The Apical Cone

The slim, smooth apical thimble at the tip of *Elodea* is distinctive. Apices of most other species that have been studied in detail are broader and generally differentiate more quickly (7, 13, 14, 15, 18). Fig. 3 is a longitudinal section of the apex under higher magnification. At the tip there is a semicircle of larger cells. These represent a hemisphere of about 20 to 25 initial cells. The cells forming the outer surface of the shoot are derived from the superficial layer of initials and are the result of divisions in the anticlinal direction.

In summarizing his work on the apical promeristem, Schmidt (13, p. 389) has brought out the following points:

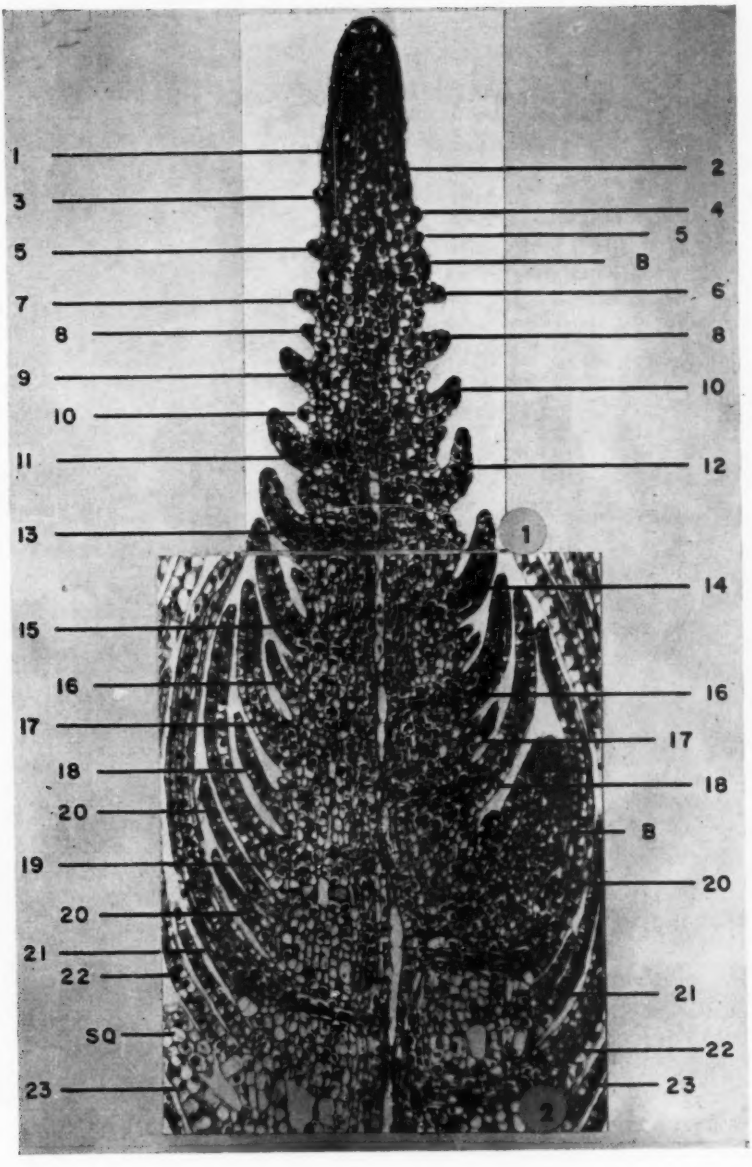
> "Architektonik des Vegetationspunktes
>
> 5. Das Urmeristem des Vegetationspunktes ist in Tunica und Corpus gegliedert.
>
> 6. Unter Tunica verstehe ich die Gesamtheit der peripherishen Schichten des Vegetationspunktes, in denen, abgesehen von der Anlage der Blätter, in der Scheitel-Region des Kegels perikline Teilung unterbleibt.
>
> 7. Das von der Tunica umschlossene Gewebe nenne ich Corpus. In ihm sind zwar auch die äusseren Zellen zu bestimmten Zeiten in einer deutlichen Schicht angeordnet, aber diese wird während eines Plastochrons in der verschiedensten Art in eine Doppellage aufgespalten."

Later workers have found that the condition is not as clear as it appeared to Schmidt and have altered the definition of the tunica to include layers

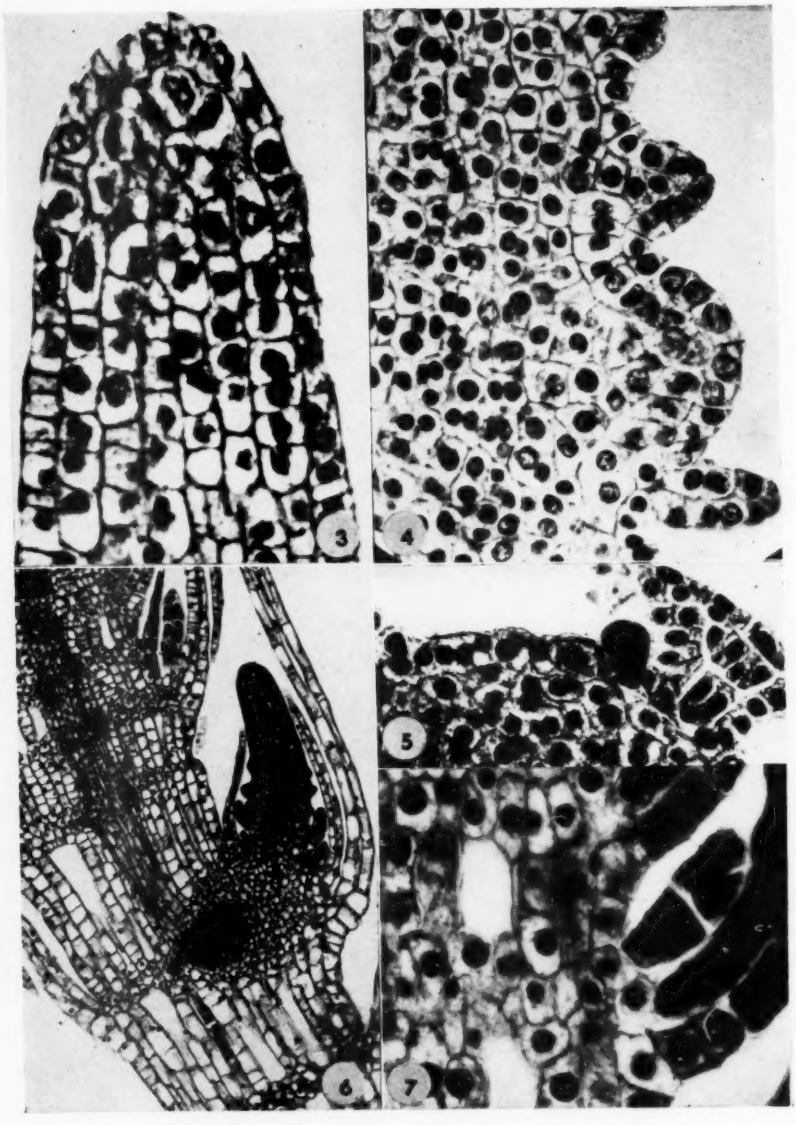
---

FIGS. 1 and 2. Median longitudinal sections of shoot, ×130.
The numbers 1 to 23 refer to the whorls to which the leaves are attached, numbered from the apex. In some cases, where the leaves have grown considerably, two or even three from the same whorl are present in the plane of the section. Only one squamule (*Sq*) and two axillary buds (*B*) are visible.

NOTE: The photomicrographs (Figs. 1–18) of *Elodea canadensis* were fixed in formalin–aceto–alcohol, dehydrated, and embedded in paraffin by the tertiary butyl alcohol method; microtomed; and stained with Delafield's haematoxylin.



Dale—Can. J. Botany


3
4
5
6
7

that also give evidence of occasional periclinal divisions. The remoteness of the leaf initiation from the promeristem in *Elodea* produces a very stable shape during plastochron. In spite of this stable shape in *Elodea densa*, which has a very similar organization, authors have differed widely in the number of layers they ascribe to the tunica, from four—the largest (8)—to one (18). Moreover they include in their discussion the whole of the apical cone down to the first leaf primordium and this includes much more than the Urmeristem of Schmidt (13) for in *Elodea* a good deal of rib meristem (14) is included in this cone. However, if only the hemisphere of apical initials is included, most median sections give the appearance of a single layered tunica. On the other hand, a section that is not through the center often has two or three layers that seem to result solely from anticlinal divisions. Below this apical group of initials the so-called rib meristem (14) proceeds to the bottom of the figure with predominantly anticlinal divisions throughout, producing vertical rows of cells. It will be noted that in the lower half of Fig. 3 the cells are arranged in orderly rows. This is the result of repeated divisions in the same anticlinal plane. The outer layer is composed of short squat cells, as if rapid cell division had not allowed for cell enlargement between divisions.

In the region of the apical cone above the first leaf primordium, air spaces are initiated as described by Hulbary (8) in *Elodea densa*. Where three or four cell walls come together in a section, dark-colored triangles are seen and occasionally a light-colored area occurs in the center of the triangle. These small chinks are the beginnings of air spaces and are seen occasionally throughout the cone, beginning within three or four cells of the tip at the upper limit of the rib meristem. With the exception of vacuolation, no further differentiation of tissue is apparent until just within one or two cells of the first leaf primordium.

2. Leaf Initiation

In *Elodea canadensis* the sessile leaves are normally initiated about 150 μ from the tip of the apex. The leaf whorl 3 in Fig. 1 is roughly 200 μ from the tip but although this is the first primordium apparent in this section, it is a representative of the third whorl of primordia. The mode of production of the primordia agrees with that of *Elodea densa* as described by Herrig (7) and Stant (18). Each leaf is discrete and separate from the rest of the whorl, and is initiated by a fold in the superficial layer after rapid division in the anticlinal direction. Two of the cells in this outer layer form the

---

FIG. 3. Median longitudinal section of stem tip, ×500.
FIG. 4. Longitudinal section, ×425. Leaf primordia from the 4th, 5th, and 6th whorls from tip. Branch primordium in the axil of leaf in 6th whorl.
FIG. 5. Transverse section, ×425. A squamule intervaginales initial appears as a darkened outgrowth from the outer row of cells in the same plane as a developing leaf initial.
FIG. 6. Longitudinal section of developing stem with branch bud and root initial, ×65.
FIG. 7. Longitudinal section, ×500; enlarging cortical lacunae; a squamule intervaginales is illustrated on the right between two developing leaf initials.

primordium of the lamella. Periclinal division of three cells in a horizontal row in the layer beneath now takes place, and the daughter cells enlarge and later divide to give rise to the midrib, the only part of the leaf more than two cells deep. The further development of the lamella is due to the division and growth of cells throughout the primordium. There is no evidence of a greater frequency of division in the basal section to correspond to the basal meristem that occurs in many monocotyledons.

Fig. 4 shows a longitudinal section of developing leaf primordia from the fourth, fifth, and sixth whorls and a branch bud in a position similar to *B*, on the right side of the apex in Fig. 1. The leaf primordium from the fifth whorl seen above the center of the figure is cut in a median plane and illustrates the beginning of midrib development. One of the cells from the second layer has divided periclinally to produce the horizontal row of cells initiating the midrib. Stant (18) points out that in *Elodea densa* not only do these periclinal divisions initiate the leaf midrib but also later anticlinal divisions of some of the progeny increase the diameter of the stem as well. She considers that this is evidence of the presence of a very reduced flank meristem (14). The lower of the two leaf primordia in the figure is not cut through the midrib and illustrates that the leaf blade is only two cells thick.

On Fig. 1, No. 9 and No. 11 illustrate the development that has occurred in the midrib at the 9th and 11th whorl of leaves. The original cells have now divided, producing a midrib two cells thick. A primordium at the 12th node, No. 12, illustrates another point in that the cells on the adaxial surface are larger than those on the abaxial, except at the base of the leaf. This difference of size is still greater in the adult leaf as shown, leaf section at the right of Fig. 16. At the base of each mature leaf much enlarged abaxial cells form a buttress, doubtless adding to its support. This is seen at the top of Fig. 16 and in longitudinal section at the right of Fig. 8.

At a level of the 12th leaf whorl (No. 12 in Fig. 1) air space tissue is initiated in the leaf. Spaces appear at the center of the triangles where three cell walls come together. In the mature leaf, these horizontal chinks running across the leaf develop little beyond this stage. Similar vertical ones appear in transverse section. They are somewhat larger at maturity and run the length of the leaf, and their appearance in the adult leaf can be seen in a leaf section towards the left side of Fig. 16. Modern workers agree with Haberlandt (6) that they are a regular feature of tissue made up of files of elongate cells.

In the axil of each leaf is a pair of structures which received their first comprehensive, though brief, treatment and name, "squamulae intervaginales", from Irmish (9). Caspary (3) published his account of "Die Hydrilleen" the same year and discussed their anatomy in *Elodea canadensis* as well as in the related genera. Their simple anatomy in *Elodea canadensis* is described as consisting of two layers of large cells devoid of chlorophyll and having no trace of vascular tissue. This lack is regarded by a later author (1) as an indication that they have a trichome nature. Caspary (3) also demon-

strated that they are generally round in face view, but may be elliptical under some conditions. He also pointed out that they appear when the leaf primordia are somewhat enlarged. In the material that I have studied they become noticeable at the level of No. 12 in Fig. 1 as the internodes are initiated. They also occur in the axil of the spathe of the male flowers. Schilling (12), who studied these structures from the point of view of function, claims that they secrete mucilage, although this may not always hold true (17). The nucleus and cytoplasm of their cells take a deep stain with haematoxylin, as can be seen from the photomicrographs, Fig. 16 between the lowest leaf and the stem, and in the center at the right between two leaves in Fig. 7.

Sanio (10) pointed out that in *Elodea canadensis* the squamules arise without connection with the leaves from the external cell layers of the apex, while Solereder (17, p. 42) speaks of them as "die sogenannten Achselschüppchen, die *Squamulae Intervaginales*, die nur trichomatische Bildungen und an der Blattbasis gelegen sind". Their development as observed in the present study is substantially that of a form of trichome. A single epidermal cell elongates so as to project beyond the epidermis, then divides. These elongate cells then divide transversely, as illustrated at the top of Fig. 5 where one is seen in the axil of a young leaf, the cytoplasm of the squamule cells having a deep stain. Subsequent cell divisions produce the squamule. Later stages can clearly be seen at the right side of Fig. 7 and again at the top center of Fig. 6. Fig. 16 shows mature stages in transverse, when the squamules have broadened.

Although they themselves are devoid of vascular tissue, their base is always adjacent to the vertical cortical strand. They are separated by only a single cell from the vertical cortical vascular system and therefore from the whole vascular tissue of the plant.

Arber (1) in her paper dealing specifically with the squamulae intervaginales of the Helobieae has claimed that they are not associated with the leaves in whose axils they appear, but rather with the internode and the leaf whorl above. However, they are initiated at a time when the internodes are only a single cell in depth and remain at the bottom of the internode, being only one cell from the horizontal and vertical vascular strands connected with the leaf, so that in development and at maturity they give the appearance of being associated with the node to which they are attached, as can clearly be seen in Figs. 5 and 7. It is also interesting to note that the number of squamules per node is determined by the leaves in whose axils they appear. Near the base of the branches the number of leaves per node increases from two to three. There are always four squamules at whorls containing two leaves and six at the whorls made up of three leaves. If they were in reality associated with the leaf whorl above, as suggested by Arber, it would be expected that there would be six squamules at a whorl of two leaves, when the whorl above has three leaves. Finally, branches are considered as nodal outgrowths occurring in the leaf axils. Squamules are situated between the leaf and the branch in its axil, again indicating that they are associated with the nearest whorl.

3. Buds and Branching

Fig. 4 shows a branch initial appearing in the axil of the sixth whorl. It can be seen that the outer layer has been pushed out by the divisions and the enlargements of more than one layer of the cells beneath. The earliest stage that it has been possible to recognize as a bud primordium has been seen in the axil of the fourth leaf whorl. In it, the bulge consists of 12 cells, mainly the progeny of the cells of the second layer. Divisions of the cells deeper in the apex occur later. The whole appearance is very different from that of the leaf primordium in which the superficial layer takes the prominent role. The further growth of the branch bud by repeated divisions produces a conical structure identical in appearance with the apical cone at the tip of the main axis. In Fig. 2 the developing cone is seen at *B* and in Fig. 6 is a more advanced stage, two millimeters from the apical tip. Note the darkened center of the section due to a root initial, and the loose parenchyma tissue connecting bud and root. The vascular tissue is not in this section. The first two leaflike scales develop on the flanks of the developing bud and elongate and grow until they enclose the tip before the bud becomes quiescent. The bud at this stage may remain dormant for some time.

4. Root Initials

The root apices are initiated at the node some distance below the stem apex. They first become apparent after the cells of the stem tissues have visibly differentiated. Their initiation takes place deep in the tissues of the stem and follows the pattern described for other adventitious roots. As far as has been seen, an initial invariably accompanies a bud and appears in the thickened nodal tissue at its base as seen in Fig. 6. Many of these root initials do not emerge from the cortex.

Differentiation

1. Nodes and Internodes

Chouard (4) has discussed the apical development in some monocotyledons. Of his second type of three he states (p. 89):

> " 'Les feuilles sont des dépendances de l'axe' *Elodea* (*Anacharis*) *canadensis* Michx. en est un bon exemple. En coupe longitudinale, on voit l'apex de la pousse formé d'un manchon cylindrique de tissus, dépourvu de tout apendice. C'est un *axe*, une *tige*. C'est même un axe réellement nu, sans feuilles, sur les 75 à 150 premiers microns (μ) de sa longueur.
>
> Plus loin de l'apex les ébauches foliaires commencent à apparaître. Ce sont de petites protubérance épidermiques, séparées l'une de l'autre, dès l'origine, par la longueur de deux ou trois cellules. On voit qu'ici *les entre noéuds existent dès l'origine.*
>
> Dans un tel cas, *la tige est l'organe primitif. Les feuilles sont seulement des appendices, des expansions, subordonnées à l'existence préalable de l'axe.*"

The evidence for the separation of the leaf primordia by two or three cells is given in his Fig. 2. This drawing is not unlike the upper part of Fig. 1, except that his cut is not median. As a result, the cone is foreshortened and the cell arrangement distorted. However, at the initiation, the leaf primordia are small, only three cells wide. Usually leaves from only half the whorls appear on each side of the apex, as indicated by their numbering in Figs. 1

and 2. And even a casual examination with dissecting microscope will reveal that the level of the developing upper epidermis at the midrib of one whorl of leaves is on the same level as the lower epidermis of the next. In serial transverse sections of numerous apices of *Elodea canadensis*, cut as fine as 5μ, there is no evidence of even a single layer of internodal primordium. The leaf insertion disks lie closely appressed until the leaves expand. As this occurs the intercalary meristem develops at 300 μ from the apex and carries the nodes apart. This is similar to the condition described in *Zea mays* (5, 15). In the initiation of the internodes the earliest stage is the elongation of cells. It is difficult to state whether these are derived from the leaf insertion disk above or below, but the cell size and arrangement suggests that it is the same as in *Zea mays*, in which the intercalary meristem is derived from the bottom of the leaf disk (5, 15). This elongation by the cells destined to become internode can be seen in Fig. 7. The cells on either side of the large opening in the cortex are longer than the corresponding nodal cells above and below. Repeated divisions of these cells, with their enlargement due to vacuolation, results in the internode. Fig. 10 shows the result of the division of cells. The cells above and below the node illustrated in Fig. 8 show the final result at lower magnification.

The nodal cells are the cells of the leaf insertion disk that do not elongate. In Fig. 10 they are distinguishable from the cells whose development has just been described, as they do not swell, enlarge, and divide but remain globose in shape. In Fig. 7 the internodal cells have elongated while the nodal have divided horizontally, whereas, in the nodal cells above and below, the division has been vertical. The nodal cells have also enlarged isodiametrically into a rounded globose shape with prominent chinks in the corners of the cells. This development has reached its conclusion in the nodal tissue forming a horizontal band in Fig. 8.

2. Vascular Tissue

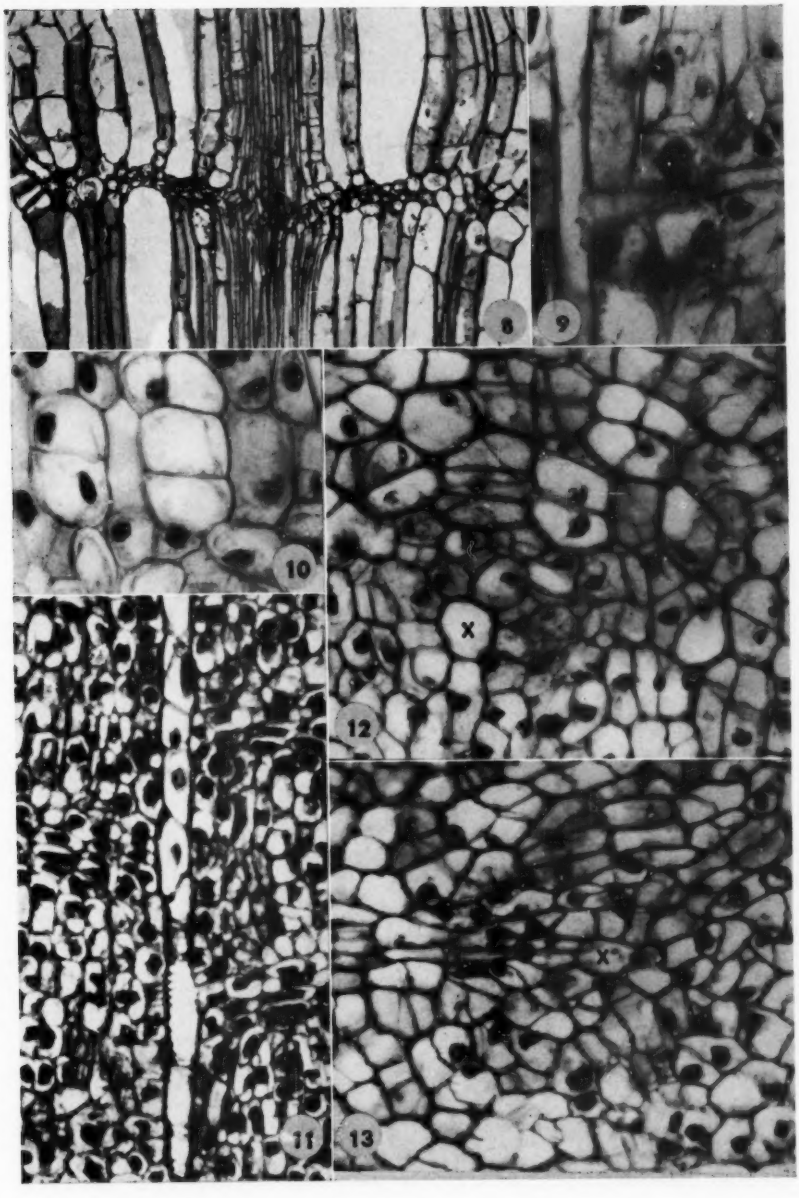
Except for the appearance of small chinks within four or five cells of the apex, the first sign of differentiation is the appearance of vascular tissue. The cells that are destined to become wood are apparent first, differentiating in the center of the stem. They enlarge greatly as a result of vacuolation. In Fig. 1 they are first seen at the height of No. 11. In the apex from which this section in the series was taken, the first sign is at the height of No. 9. Fig. 11 shows the row of cells from Fig. 2 at higher magnification. The nuclei are apparent in the upper cells although they are not as densely stained as in the surrounding cells. The cells appear more transparent because of the large vacuoles. Later stages shown lower in Fig. 11 have scalariform thickenings and have lost their protoplasm. Still lower down the stem, the cells disintegrate, first the end walls, then the complete cells with their spiral thickenings, until there is merely a hole or lacuna in place of the wood. It disintegrates not only in the internodes, but also at the nodes (6) as seen at the right of Fig. 9, which suggests that something other than tearing and

stretching is involved. Surrounding this is a strand of residual meristem that does not differentiate into the long, narrow procambial cells until later.

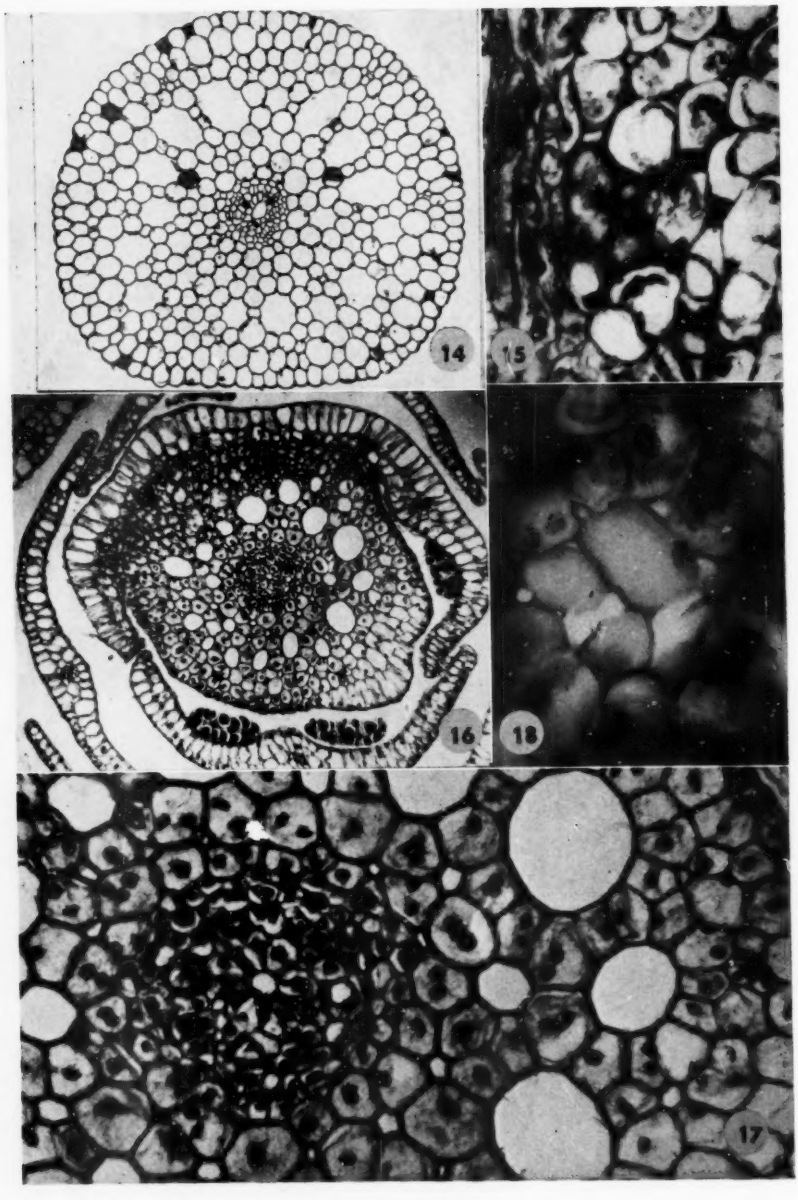
In the leaf traces the situation is different. The procambium develops before the wood differentiates and is similar to that described in most plants. The first indication in Fig. 1 is at the base of the leaf at No. 11. The procambium is clearly visible running out to the leaf at No. 14 and all other older leaf traces that are cut medially. Fig. 13 is a transverse section. The large cell marked with an '*x*' in the center is the cell that will later become xylem, but is still living. From it three strands of elongated cells, incipient procambium, radiate in a Y formation towards the three leaves of the whorl.

Fig. 12, through the adjacent internode, shows the situation with regard to the central strand. Numbers of the cells around the xylem retain their original size and shape. Others have undergone divisions which suggest the procambium condition. At this stage the procambium has not developed as far in the central strand as in the leaf trace. Fig. 11 also gives evidence of this where two procambial leaf traces can be seen extending to the right of the figure and one to the left, the latter touching the central cell. This longitudinal splitting of residual meristem into procambial cells in the cortical leaf trace appears to be contemporaneous throughout its length. Even at the very early stage shown in Fig. 13 the strand runs from the central xylem cell throughout the stem and into the base of the developing leaf. It has not been possible to decide in which direction differentiation proceeds. After it has been established in the cortical region, the process advances out into the leaf. On the upper side one or more rows of tracheids develop, beginning, as far as has been seen, at the central xylem and extending out into the base of the leaf, but not into the blade. The wood of such a leaf trace connecting up with the central strand is shown in Fig. 9 in longitudinal section and at the left side of Fig. 15 in transverse. The wood in such traces develops at a level of about the 20th whorl from the apex. At this stage the central stelar residual meristem begins to split up into procambial cells. Several nodes below this, depending on conditions of growth, the solid core of procambium surrounding the central strand of wood is complete. In the material that has been studied, no further development into sieve tubes has been seen. This is in agreement with the observations by Caspary (3) and Haberlandt (6) and disagrees with those of Schenk (11). The appearance of this region in a fairly mature transverse section is illustrated in Fig. 17. Caspary (3) described the procambial cells of the bast in some detail. Using microchemical tests he concluded that the cells contained abundant nitrogenous substances and designated them as the 'Vasa propria' of Von Mohl.

FIG. 8. Longitudinal section, ×65; mature stem with node.
FIG. 9. Longitudinal section, ×500; node with leaf trace element running to the left from central xylem lacuna.
FIG. 10. Longitudinal section, ×500; developing lacunae.
FIG. 11. Longitudinal section, ×425; enlarged part of Fig. 2 showing vascular initiation.
FIG. 12. Transverse section, ×500; developing internodal air spaces.
FIG. 13. Transverse section, ×500; developing vascular tissue at node.



Dale—Can. J. Botany



Dale—Can. J. Botany

The Casparian bands in the endodermis of the stem appear late, but sections made three or four centimeters from the apex invariably demonstrate this feature.

In addition to the vascular tissue described, *Elodea canadensis* has cortical bundles, extending vertically through the stem, the number of bundles being dependent upon the number of leaves on the whorl immediately above, two bundles associated with each leaf. The diagram, Fig. 19, illustrates the arrangement of these bundles in a stem with three leaves at each node. Three nodes and part of four internodes are represented.

Each bundle consists of three to seven previously mentioned angular phloem cells within the first cortical layer. They pass through the node on the two flanks of the leaves, just beneath the "squamules". They are connected with the leaf trace by horizontal branches of the same type of cells. These strands first appear with the intercalary cell divisions that produce the internode. Fig. 7, at the right in the second layer of the cortex, shows a very early stage in the development of a vertical strand. It has been initiated by vertical divisions in a cell that extends from one leaf base to the one above it. The horizontal cell divisions that enlarge the internode later take place in these cells as well as in the surrounding parenchyma. At the center of the extreme right of Fig. 17 a dark area shows the position of one of these strands, and in Fig. 14, sections of six of these can be seen illustrated in the outer cortex of a mature unstained internode.

3. Cortex

Hulbary (8) in his investigation of cell shape in *Elodea densa* illustrates several stages in the development of the cortical lacunae. He points out that they are schizogenous, extend from node to node, and are produced by cell divisions perpendicular to the cell walls abutting on the spaces, and he is interested in the influence of these changes on the shape of cortical cells. His observations hold true for *Elodea canadensis*, except for differences in measurements and in number and arrangement of lacunae. As pointed out earlier, small chinks, characteristic of parenchyma tissues in general, occur at the corners of the cells close to the apex. In the normally growing plant they appear at the upper limit of the rib meristem. Just below the region of rib meristem, further evidence of differentiation begins, and the cortical cells, because of their increased vacuolation, become distinguishable from residual meristem cells of the central core and leaf bases. This can be seen in Fig. 1 at a level of No. 5 and below. When the intercalary growth which results in the internodes is initiated, there is first a tendency for the cortical

---

FIG. 14. Transverse section, ×65; mature stem internode, unstained.
FIG. 15. Transverse section, ×500; developing diaphragm leaf trace elements at left.
FIG. 16. Transverse section, ×65; developing lacunae at internode.
FIG. 17. Transverse section, ×250; developing vascular tissue and internodal lacunae; enlargement of Fig. 16.
FIG. 18. Transverse section, ×500; nodal diaphragm, perforations at corners of cells.

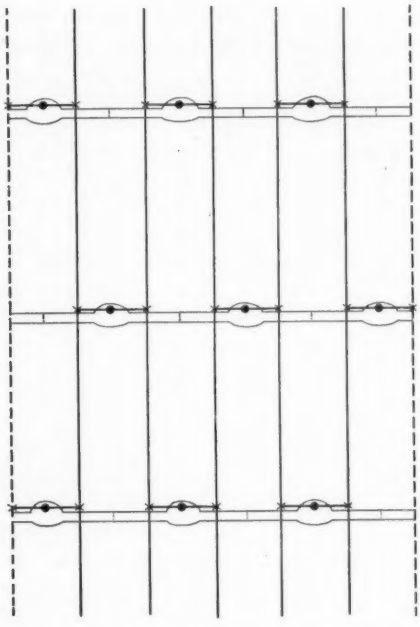


FIG. 19. This diagram represents the ramifications of the vascular strands in the cortex. The vertical dotted line at each side represents a vertical cortical strand that has been split before flattening of the cortical tissue.

The vertical lines represent the vertical cortical strands. The dark horizontal lines joining pairs of the vertical lines are horizontal strands at the nodes.

The × at each end of a horizontal line represents the position of the base of a squamule.

The black dot shows the position of the horizontal strand running from the central vascular core of the stem out into the leaf.

The thinner lines represent the position of the leaf bases at each node.

cells to elongate and then to divide by repeated transverse divisions into rows. By this time the chinks at the cell corners enlarge and coalesce and run from end to end of the cell. This stage is shown in Fig. 12 where such chinks in the cortical tissue towards the top of the figure are very evident. Subsequent to this, cell divisions are rapid and while the internodes are being produced by horizontal cell division, the nodal cells are dividing chiefly in a vertical direction, increasing the area of the stem in cross section and allowing room for the growth of longitudinal chinks into lacunae. Figs. 7 and 10 show such lacunae at an early stage in their development. In Fig. 7 the internodal cells at the sides of the lacuna are still in their elongate stage, in Fig. 10 they have divided by transverse divisions. While the divisions in the internode are predominantly transverse, longitudinal ones occur in such a direction that the walls radiate from the lacunae as mentioned by Barrett (2) and Hulbary (8). Figs. 16 and 17 are transverse sections of similar early stages. Near the center of Fig. 17 are small quadrilateral chinks and towards the

right of the figure are larger spaces surrounded by several cells with their walls radiating from them. It is evident that cell divisions in a radial direction have taken place to produce the larger number of cells in contact with the air space. The lacunae on the right of Fig. 17 illustrate the number found in the adult stem, as seen in Fig. 14. It is interesting to observe that the young lacunae, while surrounded by actively growing and vacuolating cells, are smooth in outline (Fig. 16) whereas in the mature stem the cells bulge out into the lacunae (Fig. 14). At the stage illustrated in Fig. 17 the cells are dividing actively and little elongation has taken place. The shortness of the cells accounts for the apparent presence of two or three nuclei in many cells although the section is less than 10 μ thick. After the cells stop dividing they elongate considerably and the tissue of the internode may be considered mature.

In the meantime, the nodal cells, while dividing in both anticlinal and periclinal directions, remain more or less isodiametric. The nodal tissue at an early stage is shown in Fig. 10 below the lacuna, and the small air spaces shown in this tissue do not develop further. The result is that in the adult stem, the lacunae of adjacent internodes are separated by perforated diaphragms of nodal tissue. Such tissue is seen in longitudinal section in Fig. 8 and in transverse section at an early stage in Fig. 15, and more mature in Fig. 18 where the walls of the cells have been torn in the preparation of the section. At various places in the nodal tissue, especially in the neighborhood of the central strand, the air spaces appear to be lined or partially occluded by a substance that is recognizable by its affinity for haematoxylin. This substance is responsible for the darkness in Figs. 15 and 18 and at the nodes in Fig. 8.

Acknowledgments

I should like to acknowledge my gratitude to Professor H. B. Sifton, who made it possible for me to do this work and gave generously of his knowledge throughout. My thanks are also given to Mr. Leslie Garay, who so kindly traced Fig. 19.

References

1. ARBER, A. On the "Squamulae Intervaginales" of the *Helobieae*. Ann. Botany, **37**, 31–41 (1923).
2. BARRETT, K. The origin of the endodermis in the stem of *Hippuris*. Ann. Botany, **30**, 91–99 (1916).
3. CASPARY, R. Die Hydrilleen (*Anacharideen* Endl.). Jahrb. wiss. Botan. **1**, 377–513 (1858).
4. CHOUARD, M. P. Le meristème des pousses vegetatives de quelques monocotyledones. Ann. Sci. Nat. Botan. Ser. 10, **19**, 83–92 (1937).
5. ESAU, K. Ontogeny of the vascular bundle of *Zea Mays*. Hilgardia, **15**, 325–368 (1943).
6. HABERLANDT, G. Physiological plant anatomy. Translated from 4th German edition by M. Drummond. Macmillan & Co., Ltd., London. 1914.
7. HERRIG, F. Beiträge zur Kenntniss der Blattentwicklung einiger phanerogamen Pflanzen. Flora, **107**, 327–350 (1915).
8. HULBARY, F. L. The influence of air spaces on the three dimensional shapes in cells in *Elodea* stems and a comparison with pith cells in *Ailanthus*. Am. J. Botany, **31**, 561–580 (1944).

9. IRMISH, T. Ueber das Vorkommen von Schuppen-oder haarförmigen Gebilden innerhalb der Blattscheiden bei Monocylischen Gewachsen. Botan. Ztg. **16**, 177–179 (1858).
10. SANIO, S. Einige Bemerkungen in Betreff meiner über Gefässbündelbildung geäusserten Ansichten. (Fortsetsung). Botan. Ztg. **23**, 184–187 (1865).
11. SCHENK, H. Vergleichende Anatomie der Subersen Gewachse. Bibliotheca Botan. Bd. 1, Heft 1. 1886.
12. SCHILLING, A. J. Anatomisch-biologische Untersuchungen über die Schleimbildung der Wasserpflanzen. Flora, **78**, 280–360 (1894).
13. SCHMIDT, A. Histologische Studien an Phanerogamen Vegetationspunkten. Botan. Arch. **8**, 351 (1924).
14. SCHUEPP, O. Über periodische Formbildung bei Pflanzen. Biol. Revs. Cambridge Phil. Soc. **13**, 59–92 (1938).
15. SHARMAN, B. C. Developmental anatomy of the shoot of *Zea Mays*. Ann. Botany, N.S. **6**, 245–282 (1942).
16. SIFTON, H. B. Air space tissue in plants. Botan. Rev. 11, **2**, 108–143 (1945).
17. SOLEREDER, H. Systematisch-anatomische Untersuchung des Blattes der Hydrocharitaceen. Beigt. Botan. Centr. **30**, 24–104 (1913).
18. STANT, M. Y. The shoot apex of some monocotyledons. I. Structure and development. Ann. Botany N.S. **16**, 115–138 (1952).

ON THE EXTRACTION OF ENZYMES FROM SUCCULENT PLANTS[1]

C. H. COLES[2] AND E. R. WAYGOOD[3]

Abstract

The extraction of enzymes concerned with malic acid metabolism from succulent plants, notably *Bryophyllum calycinum*, has been made possible by the introduction of a new technique. The high concentration of organic acids in the vacuoles of leaf cells was neutralized *in situ* by vacuum infiltration with ammonia solution. Thus, during maceration, the enzyme proteins are neither precipitated nor inactivated and conventional methods of enzyme purification may be applied. Further experiments have shown that whereas *B. calycinum* plants may lose their ability to fix carbon dioxide actively in the dark following exposure to short days (7½ hours) the enzymes involved in malic acid metabolism and carbon dioxide fixation in the dark are not necessarily depleted.

Introduction

Succulent plants of the family Crassulaceae and others display the unique phenomenon in the plant kingdom of accumulating, at night, relatively large quantities of organic acids, especially malic, but also citric and isocitric, etc. (4, 9, 10). These acids disappear to a large extent during daylight hours and it is this diurnal fluctuation coupled with the large turnover of acids that distinguishes them from their herbaceous counterparts. The accumulation of acid at night is associated with a respiratory quotient (R.Q.) considerably lower than unity and this has been referred to in the literature (15, 21) as the 'de Saussure effect' in honor of de Saussure, who observed the phenomenon in *Opuntia* spp. some 150 years ago.

In 1947, Thomas and Beevers (14, 16) recognized the possibility that the accumulation of malic acid in the dark in succulent plants, especially *Bryophyllum* spp., was due to the metabolic incorporation of carbon dioxide into a 3-carbon acceptor mediated by a Wood–Werkman reaction. There is now considerable evidence in favor of this hypothesis. For example, it has been shown that by increasing the carbon dioxide tension of the atmosphere surrounding the leaves, acids accumulate at a greater rate and to a larger extent and the respiratory quotients become considerably lower, often attaining negative values (5, 14, 15, 16, 18, 20), than in leaves around which the carbon dioxide tension is lower. Tracer studies with radiocarbon have shown also that carbon dioxide is incorporated into the carboxyl groups of malic and isocitric acids and that both glutamic and aspartic acid become labelled (13, 17, 18, 20). This is essentially the same pattern of tracer distribution as would be found in non-succulent plants during exposure in the dark, the difference being, however, in the magnitude of the turnover.

[1]Manuscript received August 20, 1956.
Contribution from the Department of Botany, McGill University, Montreal, Quebec. The authors gratefully acknowledge the financial assistance of the Charles F. Kettering Foundation, Yellow Springs, Ohio, U.S.A.
[2]Professor and Chairman, Department of Biology, New England College of Pharmacy, Boston, Mass., U.S.A.
[3]Professor of Botany, University of Manitoba, Winnipeg, Manitoba.

The occurrence of the necessary enzymes involved in malic acid metabolism of 'succulents' has been a matter of considerable concern and the obvious speculative standpoint has been taken (17). Either carbon dioxide is fixed into pyruvate by the oxalacetate carboxylase system with the subsequent reduction of oxalacetate to malate by a DPN-linked malate dehydrogenase, or there is a direct reductive carboxylation of pyruvate to malate catalyzed by the Ochoa TPN-linked malate enzyme. Since the recent demonstration of a phosphoenol-pyruvate carboxylase in higher plants (3) with an equilibrium in favor of the formation of its end product oxalacetate, it would be reasonable to speculate that this enzyme, which may possibly catalyze the Wood–Werkman reaction *in vivo*, would be a more likely candidate to fulfill the role of catalyzing carbon dioxide incorporation into malate in *Bryophyllum*.

Apart from some isolated reports there is a paucity of information concerning the occurrence of enzymes in succulent plants. Clendenning *et al.* (6) demonstrated the presence of oxalacetate carboxylase in *Kalanchoe* spp. Anderson *et al.* (1) reported the presence of isocitrate dehydrogenase and other enzymes in leaves of *Sedum spectabile*, and recently phosphoglyceryl kinase has been detected in the aerial portion of *Bryophyllum* spp. (2).

Undoubtedly, the unique acid metabolism of these plants has been responsible for this lack of information. The high concentration of organic acid in the leaf cells inactivates the enzyme protein during maceration or grinding of the leaf tissue. The present authors have attempted to overcome this effect by grinding the leaf tissue in the presence of alkaline phosphate and borate buffers and sodium and calcium hydroxides without success. Apparently, inactivation occurs prior to neutralization. However, by using the readily permeable ammonium ion it is possible to neutralize the acids *in situ*, which allows the extraction of much undenatured enzyme protein that can be purified by conventional methods of protein precipitation.

Experimental

Depetioled leaves were washed and vacuum infiltrated with 1% ammonia solution and allowed to stand submerged for 5 hours in the cold (4° C.). The leaves were then ground in a cold mortar with sand to which a volume of distilled water equal to their original weight had been added. The resulting brei was squeezed through nylon and the filtrate centrifuged to remove remaining debris. The supernatant (pH 9–10) was made 0.66 saturated with respect to cold ammonium sulphate and after precipitation was complete in the cold, the residue was centrifuged off, redissolved in a minimum of water, and dialyzed against distilled water overnight in the cold. Insoluble material was then centrifuged off.

This enzyme preparation contained a number of enzymes in addition to the following known to be involved in malic acid metabolism: malic enzyme, malate dehydrogenase, oxalacetate carboxylase, glutamate–oxalacetate transaminase, and isocitrate dehydrogenase. Both malic enzyme and malate dehydrogenase were determined spectrophotometrically by following the

rate of reduction of pyridine nucleotides in appropriate systems. Oxalacetate carboxylase was determined manometrically by measuring the rate of decarboxylation of oxalacetate along with essential controls. Transaminase was determined qualitatively by observing the appearance of aspartic acid on paper chromatograms after glutamic and oxalacetic acids were incubated with the enzyme preparation.

Having demonstrated the presence of these enzymes in extracts from *B. calycinum*, we thought that it would be of considerable interest to assess, if possible, the relative importance of the malic enzyme in carbon dioxide fixation in the dark as compared to a combination of oxalacetate carboxylase and malate dehydrogenase. To procure such information the first step would be to compare the enzyme complements of *Bryophyllum* leaves, which normally accumulate malate and fix carbon dioxide, with those of leaves that had lost this capacity, if these could be found.

The carbon dioxide fixing capacity of the leaf tissue in the dark was determined by measuring the respiratory quotient of leaf discs by the indirect "two vessel method" of Warburg (19). Values of the respiratory quotient below unity were considered indicative of fixation of carbon dioxide in the dark and accumulation of malic acid (15, 16).

In all experiments the leaves were collected in the early afternoon, preferably on sunny days. Discs of 10 mm. diameter were removed from the leaves by a No. 6 cork borer and washed in running tap water. Six leaf discs were placed in each of two rectangular Warburg flasks (V = ca. 20 cc.) containing 1 ml. and 7 ml. of 0.08 *M* sodium bicarbonate respectively. An atmosphere of 4% carbon dioxide in air was provided and manometer changes were recorded for a period of about 1 hour in the dark at 10° C.

Young and older leaves from a summer population of *B. calycinum* were tested in this manner and both leaf types showed respiratory quotients ranging from 0.5 to 0.6, indicating active carbon dioxide fixation. Both young and older leaves contained the full complement of enzymes referred to previously, although the concentrations were lower in the latter.

Since Bennet-Clark (4) had shown that the tempo of acid accumulation in *Sedum praealtum* was greatest in the summer months at the latitude of Dublin, the possibility was considered that the carbon-dioxide-fixing capacity of leaves of *B. calycinum* was related to the length of day to which the plants were exposed. Accordingly, one group of plants was exposed to an artificially shortened day of 7 hours during the winter months of 1953–54, while another group was treated with a day artificially lengthened to 16½ hours by 300-watt incandescent lamps. It was found that leaves of plants exposed to short days gradually lost their ability to fix carbon dioxide in the dark and after about 60 days the respiratory quotient of such leaves rose to a value above unity. On the other hand leaves from long day plants retained their capacity to fix carbon dioxide in the dark giving respiratory quotients ranging from 0.5 to 0.6 under these experimental conditions.

Of interest in this connection are the findings of the Harvard workers (8, 12) that the short day plant *Kalanchoe blossfeldiana* gradually increases its ability to fix carbon dioxide in short days, but loses it when exposed to long days. These results suggest that the underlying processes which result in this relatively large dark carbon dioxide fixation are causally related to the processes governing flowering in these plants.

A survey of the enzyme complements of leaves from short and long day *Bryophyllum* plants showed the occurrence of all the enzymes referred to previously. The results are given in Table I and, for comparison, the data are expressed as CO_2 evolved per mg. dry weight per hour. In general, short day leaves contained a lower concentration of the enzymes, although this was not consistently true. The large variations are due, most probably, to the small size of the samples, since only 100 g. of leaves was used for the extraction of the enzymes. Qualitative tests of transaminase showed a considerably lower activity in the leaves of short as compared to long day plants. Whether this is significant or whether it bears any relation to acid accumulation is not known.

The most significant feature of these data is the occurrence of all these enzymes in leaves which actively fix carbon dioxide in the dark and in those that have lost this capacity. The loss of this capacity is certainly not reflected by the total absence of any one of the enzymes which are considered to be involved. By comparison, this would be expected since leaves of herbaceous plants which do not fix carbon dioxide and accumulate acid in these proportions also contain the same enzymes (6). The complete and consistent absence of any one enzyme would have strengthened the argument for the non-participation of that enzyme or combination thereof. The controlling factors in acid accumulation, therefore, do not appear to include the deadaptation of an enzyme system, although there is always the possibility that we were not studying the essential enzyme system.

We were unable to demonstrate the presence of phosphoenol-pyruvate carboxylase in extracts of *B. calycinum* although this may have been due to our method of assay. The formation of oxalacetic acid by this system (3) was determined by coupling the system with transaminase and attempting to detect aspartic acid on paper chromatograms after incubation with glutamic acid. However, Saltman *et al.* (11), using our ammonia filtration method for extracting protein and the sensitive assay method of Bandurski and Greiner (3), was able to demonstrate the presence of phosphoenol-pyruvate carboxylase in protein preparations from *Bryophyllum* leaves.

Bandurski and Greiner (3) consider that this particular enzyme may represent more appropriately the *in vivo* catalyst of the Wood–Werkman reaction rather than the oxalacetate decarboxylase of the literature. Certainly its reaction mechanism may explain some of the experimental complexities of the oxalacetate decarboxylase system *in vitro*. And it may well be that the oxalacetate decarboxylase reaction studied here represents the reverse reaction of phosphoenol-pyruvate carboxylase.

TABLE I

THE EFFECT OF SHORT AND LONG DAY LENGTHS ON THE CONCENTRATION OF ENZYMES INVOLVED IN MALIC ACID METABOLISM OF *Bryophyllum calycinum* SALISB.

Sample No.	Enzyme concentration (mm.^{3} CO_2/g. dry wt./hr.)							
	Malic enzyme		Malic dehyd.		OAA carb.		Isocit. dehyd.	
	Short	Long	Short	Long	Short	Long	Short	Long
1	2693	4501	238	1004	1584	3748	—	—
2	713	1414	238	595	2119	5431	990	270
3	554	149	238	74	16157	3013	673	456
4	713	409	475	112	—	—	—	—

NOTE: Short = 7 hour photoperiod. Long = 16½ hour photoperiod.
Glutamate–oxalacetate transaminase } Short day, weak. Long day, active.
R.Q. (av. of a no. of samples) } Short day, 1.0–1.1. Long day, 0.5–0.6.

The results of this study do not answer the problem as to which enzyme system specifically catalyzes carbon dioxide fixation in the dark by *B. calycinum*. However they do serve to show the presence of possible mechanisms mediating this fixation. Most probably the inability of *Bryophyllum* leaves exposed to short days as well as perhaps the inability of herbaceous plants to fix relatively large quantities of carbon dioxide in the dark is not due to the absence or deadaptation of a particular enzyme system. There is, of course, the possibility that short day plants produce enzyme inhibitors which control the amount of fixation but this is unlikely since the loss of the ability to fix carbon dioxide is gradual rather than abrupt.

One cannot exclude the possibility that carbon dioxide fixation is dependent on the balance between the rate of glycolytic production of pyruvate, a related compound, and the rate of its oxidative disposal by the Krebs tricarboxylic acid cycle. If the former is high compared to the latter, pyruvate could be diverted to malic acid in much the same manner as Forward (7) has proposed that the Pasteur effect in barley is due to competition between the fermentative and oxidative systems, the latter being unable to handle the pyruvate produced by glycolysis in the face of decreasing oxygen tension. It should be possible, therefore, by accelerating the rate of disposal of pyruvate in the oxidative centers to abolish dark carbon dioxide fixation by leaves of *Bryophyllum* plants exposed to long days.

In conclusion the authors wish to bring to the attention of interested laboratories the use of the ammonia infiltration method for the extraction of active enzyme protein from leaves of *B. calycinum*. This technique may well be successfully applied to other plant tissues which normally contain a high proportion of organic acid and generally resist the application of conventional enzyme extraction procedures.

References

1. ANDERSON, D. G., STAFFORD, H. A., CONN, E. E., and VENNESLAND, B. The distribution in higher plants of triphosphopyridine nucleotide-linked enzyme systems capable of reducing glutathione. Plant Physiol. **27**, 675–684 (1952).
2. AXELROD, B. and BANDURSKI, R. S. Phosphoglyceryl kinase in higher plants. J. Biol. Chem. **204**, 939–948 (1953).
3. BANDURSKI, R. S. and GREINER, C. M. The enzymatic synthesis of oxalacetate from phosphoryl-enolpyruvate and carbon dioxide. J. Biol. Chem. **204**, 781–786 (1953).
4. BENNET-CLARK, T. A. The role of the organic acids in plant metabolism I. New Phytologist, **32**, 37–71 (1933).
5. BONNER, W. and BONNER, J. The role of carbon dioxide in acid formation by succulent plants. Am. J. Botany, **35**, 113–117 (1948).
6. CLENDENNING, K. A., WAYGOOD, E. R., and WEINBERGER, P. The carboxylases of leaves and their role in photosynthesis. Can. J. Botany, **30**, 395–409 (1952).
7. FORWARD, D. F. The respiration of barley seedlings in relation to oxygen supply. II. A metabolic interpretation. New Phytologist, **50**, 325–356 (1952).
8. GREGORY, F. G., SPEAR, I., and THIMANN, K. V. The interrelation between CO_2 metabolism and photoperiodism in *Kalanchoe*. Plant Physiol. **29**, 220–229 (1954).
9. PUCHER, G. W., LEAVENWORTH, C. S., GINTER, W. D., and VICKERY, H. B. Studies in the metabolism of crassulacean plants: The diurnal variation in organic acid and starch content of *Bryophyllum calycinum*. Plant Physiol. **22**, 360–376 (1947).
10. PUCHER, G. W. and VICKERY, H. B. On the identity of the so called crassulacean malic acid with isocitric acid. J. Biol. Chem. **145**, 525–532 (1942).
11. SALTMAN, P., LYNCH, V. H., and STILT, C. The dark fixation of CO_2 by succulent leaves: The first products. Plant Physiol. Suppl. **30**, XXII (1955).
12. SPEAR, I. and THIMANN, K. V. The interrelation between CO_2 metabolism and photoperiodism. II. Effect of prolonged darkness and high temperature. Plant Physiol. **29**, 414–417 (1954).
13. STUTZ, R. E. and BURRIS, R. H. Photosynthesis and metabolism of organic acids in higher plants. Plant Physiol. **26**, 226–243 (1951).
14. THOMAS, M. Plant physiology. 3rd ed. J. & A. Churchill, Ltd., London. 1947. p. 314 ff.
15. THOMAS, M. CO_2 fixation and acid synthesis in Crassulacean acid metabolism. Symposia Soc. Exptl. Biol. **5**, 72–93 (1951).
16. THOMAS, M. and BEEVERS, H. Physiological studies on acid metabolism in green plants. II. Evidence of CO_2 fixation in Bryophyllum and the study of diurnal variation of acidity in this genus. New Phytologist, **48**, 421–447 (1949).
17. THOMAS, M. and RANSON, S. L. Physiological studies on acid metabolism in green plants III. New Phytologist, **53**, 1–30 (1954).
18. THURLOW, J. and BONNER, J. Fixation of atmospheric CO_2 in the dark by leaves of Bryophyllum. Arch. Biochem. **19**, 509–511 (1948).
19. UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. Manometric techniques in tissue metabolism. Burgess Publishing Company, Minneapolis, Minn. 1949.
20. VARNER, J. E. and BURRELL, R. C. Use of C^{14} in the study of the acid metabolism of Bryophyllum calycinum. Arch. Biochem. **25**, 280–287 (1950).
21. WOLF, J. Beiträge zur Kenntnis der Säurestoffwechsels sukkulenter Crassulaceeu. VI. Mitteilung neuere Vorstellungen vom Chemismus des Säurestoffwechsels. Planta, **37**, 510–534 (1949).

A TECHNIQUE FOR THE STERILIZATION AND INOCULATION OF WOOD CUT FROM LIVING TREES[1]

J. T. BASHAM[2]

Abstract

A method of obtaining sterilized wood blocks much less drastic than those presently in common use has been developed. The treatment is sufficiently mild that only the most superficial of the living parenchyma cells are killed. Samples of balsam fir wood treated by this technique have been successfully inoculated in specially designed tubes with three fungi that attack recently killed balsam in nature. The technique shows promise of reducing the problem of correlating field and laboratory studies of wood decay by avoiding the marked changes in the wood which result from most sterilization methods.

Introduction

There are numerous examples in the field of plant pathology of metabiotic series in which one organism replaces another in an expanding symptomological picture. In timber and forest pathology the isolation of a fungus from stained or decayed wood does not in itself constitute proof that the stain or decay has been caused by that organism. There always exists the possibility that other fungi were partly or entirely responsible for the abnormal condition of the wood. For this reason in any study of wood decay and its cause it is advisable to verify the isolation techniques through the application of Koch's third and fourth rules of proof of pathogenicity (2). In considering this problem, Nordin (3) has proposed a modification of the third postulate as follows: "Wood blocks cut from freshly felled trees must be inoculated with the fungus and the characteristic stain or decay must develop". In addition to establishing the decay potential of the organism in question, this procedure affords an opportunity to observe the effect of the fungus upon the wood without the aid or interference of other organisms.

The technique used by forest and timber pathologists to isolate fungi from stained or decayed wood is well established and universally accepted. Various techniques, however, are used in the inoculation of uninfected wood. The direct inoculation of living trees has generally proved unsatisfactory because the method is often difficult, subject to contamination and interference, and always time-consuming. Although surface sterilization by the exposure of wood blocks to fumes of glacial acetic acid, hydrofluoric acid, and other chemicals has occasionally been effective (1), the procedure is long and tedious. Furthermore, very little is known about the effect of these fumes upon the chemical composition of the wood. Another widely used technique is that of autoclaving wood blocks for periods of 10 minutes or longer at high pressures and temperatures. Tests conducted by the author and others (4) indicate that this treatment can lower the pH of the

[1]Manuscript received August 28, 1956.
Contribution No. 329 from the Division of Forest Biology, Science Service, Department of Agriculture, Ottawa.
[2]Laboratory of Forest Pathology, Southern Research Station, Maple, Ontario.

wood and break down some of its less soluble compounds into their more soluble and more easily assimilable constituents. Chemical changes of this nature in the wood substrate throw considerable doubt upon the reliability of this technique as a criterion in defining pathogenicity, and even more in estimating the decay potential of an organism.

The value of *in vitro* decay studies would be considerably enhanced by the development of a method of sterilization which leaves the wood virtually unchanged. Since wood is complex chemically it is difficult to estimate either the nature or extent of changes due to various treatments. It was decided that as a criterion for a satisfactory method the bulk of the living parenchyma cells of sound wood must be alive at the end of the sterilization procedure. The initial attack of fungi upon wood in that condition would be quite similar to an attack on the wood of living trees. There appears to be no way of avoiding the earlier death of the tissues and other changes inherent in the isolation of portions of living tissue. However, in investigations of fungi that attack only dying or recently killed trees in nature, prolonged life of the cells would be detrimental.

Sterilization of Wood Beams

A quick, simple method of sterilizing non-diseased wood freshly cut from living balsam fir, *Abies balsamea* (L.) Mill., with little or no apparent chemical, physical, or physiological effect upon the wood, is described below. This method has been developed by the author following discussions with Dr. H. M. Good of Queen's University. The technique has proved effective and has been used to sterilize balsam fir wood prior to its successful inoculation with three fungi which frequently attack and colonize recently killed balsam in nature.

A living, healthy tree is selected, felled, and the trunk cut in 10-in. bolts. From these bolts small test beams, approximately $\frac{3}{8}$ in. $\times$ $\frac{3}{8}$ in. $\times$ 7 in., are manufactured, first roughly with a band saw, then more accurately with a jointer.

The surfaces of some beams become contaminated in this process, despite frequent swabbings of the machine tools with alcohol. It is necessary, therefore, to immerse each finished beam as soon as possible in 70% ethyl alcohol for approximately 2 minutes. The beams are removed from the alcohol bath with sterilized forceps, shaken gently in the air for about 10 seconds to remove excess alcohol, then placed in sterile inoculation tubes. The remaining alcohol evaporates from the beams within 24 hours.

Beams sterilized in this manner have been placed directly on malt agar and left for as long as 4 months with no evidence of the growth of any organism on the media, indicating that they contain no fungal population.

Radial sections 20 μ thick were removed from the surface of beams cut from living balsam fir sapwood 48 hours after they had been immersed for 2 minutes in 70% alcohol. Microscopic plasmolysis tests were conducted on these sections. It was demonstrated that the ray cells in these sections

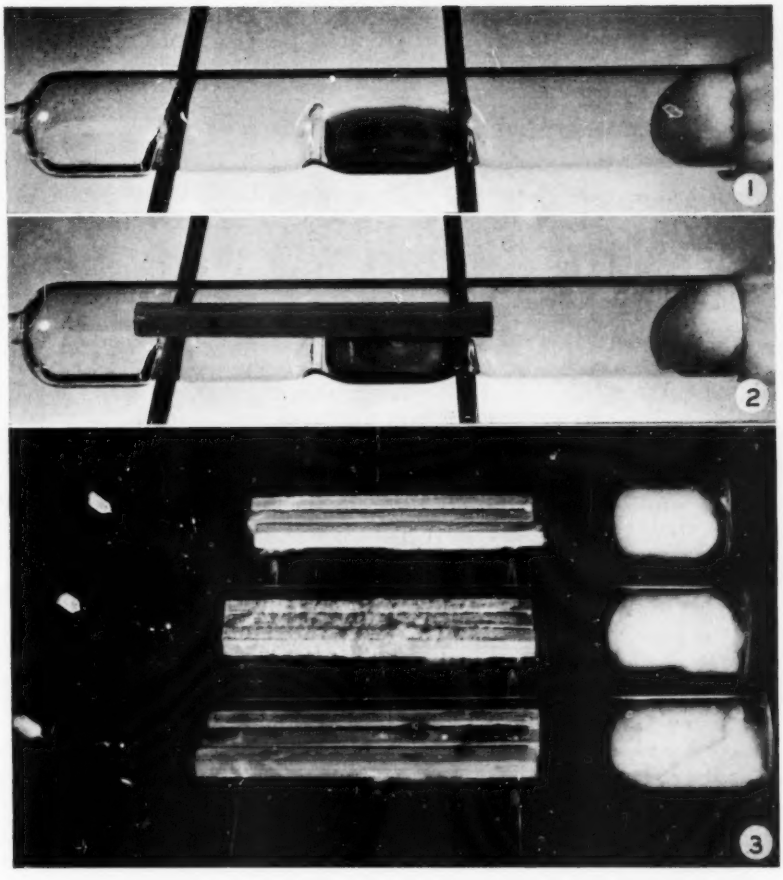


FIG. 1. Inoculation tube with agar surface covered by mycelium of *Polyporus abietinus*. The left compartment contains sterilized, distilled water.

FIG. 2. The same tube shown in Fig. 1, with the addition of a sterilized test beam of balsam fir sapwood.

FIG. 3. Three inoculation tubes photographed 14 weeks after three sterilized test beams of balsam fir sapwood were placed in each tube. The fungi, from top tube to bottom tube are *Stereum chailletii*, *Polyporus abietinus*, and an unidentified member of the Fungi Imperfecti.

can be plasmolyzed in 0.6 *M* solutions of sodium and calcium chlorides and deplasmolyzed on the addition of water. It is evident that the alcohol immersion treatment does not kill or seriously affect the ray parenchyma of these surface sterilized beams.

Inoculation of Wood Beams

An inoculation tube has been designed (Fig. 1) to support sterilized wood beams a short distance above agar media so that the wood does not touch the agar, but can contact mycelium growing upon that agar. This arrangement precludes the possibility that the chemical composition of the wood might be altered by the absorption of substances from the agar. The illustrations in Plate I show clearly how the tubes are separated by three ridges into four compartments, also the small $\frac{3}{8}$ in. diameter tube at the "closed" ends.

Tubes which are to be used are plugged at both ends with cotton and autoclaved at 15 lb. pressure for 15 minutes. Autoclaved agar is then poured into the second compartment almost to the tops of the ridges, using a sterilized right-angled piece of glass tubing. The agar is allowed to cool and solidify, and sterilized distilled water is introduced with a pipette into the fourth compartment through the $\frac{3}{8}$ in. tube. The fungus to be tested is now placed on the surface of the agar. The three fungi that have been used in these experiments covered the surface of the agar with luxurious mycelial mats within 10 days (Fig. 1). Sterilized wood beams can now be inserted in the tubes to rest upon the ridges and contact the mycelium (Fig. 2).

Excessive moisture losses through the cotton plugs were encountered in the first series of tests. This allowed the agar to dry up and shrink to such an extent that the mycelium lost contact with the beams before it could become established on the wood. This difficulty was overcome in succeeding tests by sealing the large tube openings with cigarette paper glued to the rims of the tubes with corn syrup.

Results

The technique described herein has been used with wood from one tree species, balsam fir, and with the three fungi most frequently associated with the deterioration of this species in nature. The three fungi are *Polyporus abietinus* Dicks. ex Fries, *Stereum chailletti* Pers., and an unknown fungus of the Fungi Imperfecti, probably belonging to the family Ceratostomellaceae. The extent to which these fungi have grown over the surface of the test beams 14 weeks after the sterilized beams were placed in the inoculation tubes is illustrated in Fig. 3. Upon removal of the surface mycelium, these beams resembled in appearance the wood in recently killed balsam trees from which each of the fungi was originally isolated. All three fungi have been reisolated from the central portions of those test beams they have infected *in vitro*, thereby fulfilling Koch's fourth rule of proof of pathogenicity.

Discussion

The aim of the work outlined in this paper has been to develop a technique whereby wood can be inoculated by fungi in the laboratory under conditions which relate as closely as possible to those that exist when these same fungi attack living or dead trees in the forest. This objective has to some extent been attained through the use of a wood sterilization method which leaves the wood practically unaltered, in contrast to the standard autoclaving technique.

Numerous tests have supplied convincing evidence that wood from healthy, living, balsam fir in which no visible signs of fungal action can be observed is entirely free of fungi. However, since there is a controversy concerning the existence of fungi in the healthy wood of certain living trees, it should be noted that it is unlikely that the surface sterilization method described herein would eliminate these fungi. Clearly this would be an advantage, since any sterilization technique which killed these organisms would serve only to widen the gap between laboratory conditions and field conditions of infection.

The success which has been obtained, through the use of the technique described in this paper, in the artificial inoculation of balsam fir sapwood with fungi that deteriorate this species in nature, suggests that a similar technique may be profitably used in laboratory studies of the effects of other fungi causing heart rot and deterioration in their respective hosts.

Acknowledgments

The author is indebted to Dr. H. M. Good, Queen's University, Kingston, in whose laboratory the initial work on this problem was done; to Dr. L. T. White, Officer-in-Charge of the Forest Pathology Laboratory, Maple; and to others at the Maple Laboratory and at Queen's University who have contributed to this work.

References

1. FRITZ, C. W. Brown stain in pine sapwood caused by *Cytospora* sp. Can. J. Botany, **30**, 349–359 (1952).
2. KOCH, R. Über die Milzbrandimpfung. Eine Entegegnung auf den von Pasteur in genf gehaltenen Vortrag. *Reprinted 1912 in* Gesammelte Werke. Leipzig, **1**, 207–231 (1882).
3. NORDIN, V. J. Uniformity in procedures for the culturing of decay fungi from living trees. (Mimeographed report). Canada, Department of Agriculture, Science Service, Forest Biology Division, Calgary, Alta. 1956.
4. SORLEY, D. H. Relative rates of decay of stained and normal maple wood, *Acer saccharum* Marsh. Unpublished report. Queen's University, Kingston, Ontario. 1956.

SEDIMENTARY CHLOROPHYLL DEGRADATION PRODUCTS IN SURFACE MUDS FROM CONNECTICUT LAKES[1]

J. R. VALLENTYNE[2] AND DENNIS F. CRASTON

Abstract

Amounts of sedimentary chlorophyll degradation products were determined for surface sediments from nine Connecticut lakes. The relationship between sedimentary chlorophyll degradation products in the ignitable matter of the mud and the hypolimnetic oxygen deficit was not significant at the 5% level, nor was the relationship between the former and the average summer seston chlorophyll. Shallow water sediments tended to have lesser amounts of sedimentary chlorophyll degradation products than deep water sediments.

Introduction

It is characteristic of both marine and freshwater sediments that organic solvent extracts are almost invariably colored yellow-green owing to the presence of carotenoids and chlorophyll degradation products. The work reported here was concerned only with the green-colored pigments. Vallentyne (1955 (3)) reviewed earlier work on the chlorophyll degradation products of sediments and also developed a quantitative method for their assay.

The amounts of chlorophyll degradation products in lake mud probably represent only a fraction of the chlorophylls initially present in the plants that gave rise to the mud organic matter. In fact, most of the original chlorophylls are likely converted to spectroscopically unrecognizable products before the sinking phytoplankton arrives at the mud surface. Some of the breakdown products, however, have a structure sufficiently chlorophyll-like to retain the characteristic absorption spectra of chlorophylls. It is this latter group of green-colored degradation products that can be quantitatively measured (on a relative scale) in mud extracts. The complex of green degradation products (at least four in number) will hereafter be referred to as sedimentary chlorophyll degradation products (SCDP).

Our original motive in studying SCDP was based on the hope that it might be possible to use these materials as biochemical fossils in a quantitative sense, and thus provide data bearing on the abundance of aquatic plants in lakes during postglacial time (Vallentyne 1955 (3)). There are many factors that might invalidate such an interpretation. The most obvious of these is variability in the percentage decomposition of chlorophylls and SCDP in different lakes and at different periods in the history of a given lake. The present investigation was undertaken to determine if the amounts of SCDP in a unit weight of mud organic matter were directly related to the amount of seston chlorophyll in a unit volume of overlying lake water. Our reasoning

[1]Manuscript received September 7, 1956.
Contribution from the Department of Biology, Queen's University, Kingston, Ontario.
[2]Present address: Geophysical Laboratory, Carnegie Institution of Washington, Washington, D.C.

was that there should be a parallel between the quantities of seston and mud "chlorophylls". Since rooted aquatic plants contain relatively more supporting (non-chlorophyll-bearing) tissues than phytoplankters, the former should not contribute as much SCDP to the mud as the phytoplankters, except in excessively shallow lakes clogged with rooted vegetation. It is probably reasonable to neglect the contribution from terrestrial plants except in lakes of very small area.

Materials and Methods

Sediment samples were collected from the following Connecticut lakes during August, 1955: Ball Pond, Bashan Lake, Beseck Lake, Dooley Pond, Lower Linsley Pond, Lake Pocotopaug, Pataguanset Lake, Lake Quassapaug, and Rogers Lake. Locations and other pertinent data for these lakes are listed by Deevey (1940 (1)). All samples were collected with a 6 in. Ekman dredge. The dredge samples were thoroughly mixed before an aliquot was taken for analysis. Samples were stored in glass jars at 4° C. in the dark from the time of collection to analysis. Preservative was not added at any time.

The sediments were analyzed for SCDP following the method outlined by Vallentyne (1955 (3)). Some extracts were too low in color for a reliable determination of SCDP. In those cases the extracts were concentrated and the amount of dimethylaniline added to the extraction solvent (90% acetone) was reduced from 0.5 to 0.2%. The visible absorption spectra of the 90% acetone extracts were determined with a Beckman DK-2 recording spectrophotometer. Occasional duplicates showed excellent agreement. Only in the case of the Lake Pocotopaug samples was there significant absorption above 700 mμ due to background materials. The SCDP values for the Lake Pocotopaug samples were not corrected for background absorption and may accordingly be somewhat high. None of the extracts had a 750 mμ peak (indicative of bacteriochlorophyll). Ignitable matter was determined by ashing the samples over a Bunsen burner.

Results

The spectra of the mud extracts were all similar to the absorption spectrum shown by Vallentyne (1955 (3)). There were always two main absorption maxima, one at 412 mμ and the other at 667–670 mμ, a small maximum near 610 mμ, and humps at 450, 477, and 538 mμ. The positions of the maxima and humps seldom departed more than 2 mμ from the wave lengths listed above. Only in one case was there an additional maximum (in a sample collected at 1 m. depth from Lake Quassapaug). The additional maximum occurred at a wave length of 432 mμ, i.e., the wave length at which chlorophyll *a* shows maximal absorption. It seems reasonable that this peak was due to chlorophyll *a*, derived from living plants caught in the dredge sample.

The ratios of the density readings at 412 and 668 mμ varied from 3.1 to 7.8 for different samples. One sample from Lake Quassapaug had a ratio

of 2.0 for these two peaks, but the low value was due to high background absorption. There was a slight tendency for the density ratio to increase with the depth of the sediment in most lakes. The value of the ratio is primarily determined by two factors: (1) the amount of carotenoids relative to SCDP; and (2) varying proportions of different individual SCDP. Brown and Vallentyne (in manuscript) have isolated from lake sediments four different SCDP with the low wave length absorption maximum at 411, 425, 440, and 464 mμ respectively, using acetone as the solvent. It is not yet possible to separate the influence of the two factors listed above on the ratio of density readings at 412 and 668 mμ, so we cannot draw any conclusions about the relative amounts of carotenoids present. The average values for the density ratio in the lake sediments were as follows: Bashan (3.8), Quassapaug (3.8), Pataguanset (3.9), Rogers (4.2), Beseck (4.5), Ball Pond (4.7), Pocotopaug (4.7), Dooley Pond (5.3), and Lower Linsley Pond (5.7).

The lakes studied may be divided into three classes on the basis of depth and SCDP values: (1) shallow lakes; (2) deep lakes in which the amount of SCDP increased with depth; and (3) deep lakes in which the amount of SCDP was independent of depth. Dooley Pond and Beseck Lake lie in the first class. The data for Dooley Pond are given in Table I. The amount of SCDP per g. ignitable matter is seen to be essentially independent of depth. This was also true of the samples from Beseck Lake. Data for the latter lake are not reported in detail because the results were virtually identical with those from Dooley Pond. The average ignitable matter content of eight samples from Beseck Lake was 18%. The average value for SCDP per g. ignitable matter was 54.4 SCDP units, with a standard deviation of $\pm$19.0 units. The sediments of Dooley Pond were studied for two reasons: firstly, as an example of a shallow body of water, and secondly

TABLE I

IGNITABLE MATTER AND SEDIMENTARY CHLOROPHYLL DEGRADATION PRODUCTS (SCDP UNITS PER G. IGNITABLE MATTER) FOR EKMAN DREDGE SAMPLES FROM DOOLEY POND, CONNECTICUT. THE STANDARD DEVIATION IS LISTED FOR THE AVERAGE

Depth (m.)	Ignitable matter (%)	SCDP (units per g. ignitable matter)
2	16.4	69.0
2	16.4	61.0
2.5	16.4	58.0
2.5	17.5	81.2
3	18.3	45.3
3	20.1	61.2
3	19.3	53.8
3.5	18.7	44.4
4	21.4	56.6
4	20.4	64.7
		Average = 59.5 $\pm$ 10.9

because Deevey (1940 (1)) recorded an exceptionally high amount (58.4 mg./m.^{3}) of seston chlorophyll in the surface water of Dooley Pond. While we anticipated finding correspondingly high amounts of SCDP in the mud, our expectation was not realized, as will be apparent when data for other lakes have been presented. Although the summer average for seston chlorophyll in Beseck Lake (13.56 mg./m.^{3}) was much smaller than in Dooley Pond, the value approached that found for the highly productive Linsley Pond (average summer seston chlorophyll = 15.25 mg./m.^{3}). The amounts of SCDP in the mud of both Dooley Pond and Beseck Lake were low as compared with the amounts in deeper lakes having lower standing crops of phytoplankton. In addition, it should be noted that although Dooley Pond averaged five times as much phytoplankton per unit volume of water as Beseck Lake, the amounts of SCDP in the mud were almost identical.

The data for Linsley Pond are reported in Table II, and may be taken as typical (together with the data for Ball Pond and Lake Pocotopaug) of the class of lakes in which SCDP values increased with depth. The data for Ball Pond (12 samples ranging from 3 to 13.5 m. depth) were essentially identical with those for Linsley Pond. This was true for the percentage of ignitable matter, the amount of SCDP per g. ignitable matter, and the enrichment of SCDP in deep sediments. The samples from Lake Pocotopaug (11 samples ranging from 1.7 to 10 m. depth) were lower both in ignitable matter (averaging 18% of the dry weight) and SCDP (averaging 44.9 ± 17.2 SCDP units per g. ignitable matter) but there was an increase in the amount of SCDP with depth. We shall not discuss the relation between seston chlorophyll and SCDP in these lakes until the data for other deep water lakes have been presented.

TABLE II

IGNITABLE MATTER AND SEDIMENTARY CHLOROPHYLL DEGRADATION PRODUCTS (SCDP UNITS PER G. IGNITABLE MATTER) FOR EKMAN DREDGE SAMPLES FROM LOWER LINSLEY POND, CONNECTICUT

Depth (m.)	Ignitable matter (%)	SCDP (units per g. ignitable matter)
3	53.7	51.8
3.5	29.2	97.9
4.5	22.5	83.1
5.5	47.7	78.6
6.5	27.7	140.0
7	41.3	91.7
9	35.0	104.0
9	33.5	102.0
10	40.6	121.1
10	23.7	166.0
11	33.9	118.5
12	37.6	105.0
12.5	39.7	155.0
13	33.9	118.0
13	35.0	86.6

Two of the lakes studied (Pataguanset and Bashan) showed little change in the amount of SCDP with depth. The values for Pataguanset Lake are presented in Table III. The sediments of Bashan Lake (nine samples varying from 4 to 13.5 m. in depth) averaged 22% ignitable matter for depths greater than 6 m. Shallower sediments were less organic, the amount of ignitable matter ranging as low as 2%. The average amount of SCDP in Bashan Lake was 56.1 SCDP units per g. ignitable matter, with a standard deviation of ±19.1 SCDP units.

TABLE III

IGNITABLE MATTER AND SEDIMENTARY CHLOROPHYLL DEGRADATION PRODUCTS (SCDP UNITS PER G. IGNITABLE MATTER) FOR EKMAN DREDGE SAMPLES FROM PATAGUANSET LAKE, CONNECTICUT. THE STANDARD DEVIATION IS LISTED FOR THE AVERAGE

Depth (m.)	Ignitable matter (%)	SCDP (units per g. ignitable matter)
3	27.6	86.3
4	26.5	76.5
4.5	24.9	84.8
5	26.9	77.2
5.5	27.4	81.3
6	27.0	87.9
6.5	28.7	85.1
8	30.2	85.9
8	29.5	89.4
8.5	29.9	90.0
8.5	30.3	73.4
9	29.9	84.8
9	29.6	74.7
		Average = 82.9 ± 5.4

TABLE IV

IGNITABLE MATTER AND SEDIMENTARY CHLOROPHYLL DEGRADATION PRODUCTS (SCDP UNITS PER G. IGNITABLE MATTER) FOR EKMAN DREDGE SAMPLES FROM ROGERS LAKE, CONNECTICUT. THE STANDARD DEVIATION IS LISTED FOR THE AVERAGE

Depth (m.)	Ignitable matter (%)	SCDP (units per g. ignitable matter)
10	31.2	63.0
11	32.1	59.4
11.5	32.8	56.5
12	32.0	65.4
12	29.2	65.5
13	27.0	65.5
16	30.4	74.9
17	32.7	74.0
18	30.4	90.4
18	31.5	72.0
		Average = 68.7 ± 9.2

The deep water sediments of Rogers Lake were sampled in order to assess the effect of increasing depth in the hypolimnion on SCDP values. These data are presented in Table IV. One will note a slight and barely significant increase in the amount of SCDP with increasing depth. The data are otherwise quite uniform, more so than was the case for hypolimnetic sediments from Linsley Pond.

Let us now compare the amounts of SCDP per g. ignitable matter in the hypolimnetic sediments of the six lakes that were sufficiently deep to have a hypolimnion. For any lake in temperate latitudes that possesses a well-defined hypolimnion, we can safely assume that the temperature of the hypolimnetic mud has not varied beyond the limits of 4° and 10° C. since the mud was deposited. Using only hypolimnetic muds, we can thus compare the amounts of SCDP on a temperature-independent basis.

Data for two of the measurements made by Deevey (1940 (1)) can instructively be compared to the average amounts of SCDP in hypolimnetic mud. The first of these is the hypolimnetic oxygen deficit (expressed as mg. of dissolved oxygen removed per day from a column of hypolimnetic water 1 sq. cm. in cross-sectional area). The value of the hypolimnetic oxygen deficit provides a measure of the amount of aerobic decomposition of organic matter in the hypolimnion in unit time. The value is generally assumed to be proportional to the productivity of the water body in question. The second measurement in which we are interested is the average seston chlorophyll value for surface water in the summer. Both these data are presented in Table V, together with average SCDP values for the hypolimnetic muds. It will be apparent that there is no precise linear relation between any two of the three variables (oxygen deficit, seston chlorophyll, SCDP). The correlation coefficients between oxygen deficit and seston chlorophyll, oxygen deficit and SCDP, and seston chlorophyll and SCDP were 0.689, 0.600,

TABLE V

AVERAGE AMOUNT OF SEDIMENTARY CHLOROPHYLL DEGRADATION PRODUCTS (SCDP UNITS PER G. IGNITABLE MATTER) FOR SAMPLES GREATER THAN 8.5 M. DEEP FROM SIX CONNECTICUT LAKES. DATA FOR TWO 8.5 M. SAMPLES FROM PATAGUANSET LAKE ARE INCLUDED.
STANDARD DEVIATIONS ARE LISTED FOR THE SCDP AVERAGES.
DATA FOR OXYGEN DEFICIT AND SESTON CHLOROPHYLL FROM DEEVEY (1940 (1))

Lake	Number of samples	Average SCDP (units/g. ignitable matter)	O_2 deficit (mg./cm.^{2}/day)	Seston chlorophyll (mg./m.^{3})
Pocotopaug	3	46.9 ± 17.7	11.9	6.37
Bashan	5	49.9 ± 12.0	12.8	2.08
Rogers	10	68.7 ± 9.2	31.0	2.87
Pataguanset	4	80.7 ± 7.0	20.2	4.07
Linsley Pond	9	119.6 ± 24.0	41.8	15.25
Ball Pond	7	120.6 ± 28.6	18.2	5.54

and 0.622, respectively. None of the correlation coefficients was significant at the 5% level. In other words, there is no statistically significant relation between any two of the three variables. On the other hand, it would be fallacious to conclude that the amounts of SCDP vary independently of oxygen deficit and seston chlorophyll. It would rather appear that other factors, not assessed in the present study, must be taken into account. One of these is the variability in percentage decomposition of the chlorophyll initially present in the plankton.

Discussion

The similarity of the absorption spectra for extracts from the sediments of the nine lakes studied suggests that there were no drastic differences in the proportions of different SCDP present. Bacteriochlorophyll-like materials were not present in the surface muds, as evidenced by the lack of an absorption maximum at 750 mμ. Both these results are interesting when compared with data previously accumulated for older sediments taken from core samples. Vallentyne (1955 (3)) reported that the extracts of subsurface sediments in Little Round Lake, Ontario, had an absorption maximum at 663 mμ as compared to 667 mμ for surface sediments from the same lake. Although this is the only lake in which such a change has been observed, it is notable that none of the surface sediments from Connecticut lakes showed similar decreases in the position of the upper wave length maximum. The senior author has also noted absorption peaks at 750 mμ in the extracts of some buried sediments that ranged in age from recent to 11,000 years old, which may possibly be taken to indicate that green-pigmented bacteria are active in buried deposits.

Since the quantitative data for SCDP do not show a statistically significant correlation either with Deevey's (1940 (1)) data for seston chlorophyll or hypolimnetic oxygen deficit, one must be careful in interpreting SCDP data in terms of phytoplankton abundance or productivity. If more lakes had been examined it is possible that a significant correlation might have emerged, but the brief collecting trip to Connecticut did not permit a more extensive collection of samples.

Decomposition factors must be considered in future studies. The low values for SCDP in the mud of the two shallow lakes studied, and the low values for SCDP in the shallow water of three of the deep lakes, suggest one of two possibilities: either the ignitable matter of shallow sediments is initially low in SCDP or the breakdown rates there are greater than in deep sediments. The latter is probably true for Dooley Pond since the phytoplankton contributes most of the chlorophyll to the mud in that lake. In the other lakes the organic matter of shallow deposits may be mostly derived from the supporting tissues of rooted plants.

By restricting future study to hypolimnetic muds one could negate the influence of temperature on SCDP decomposition *in the mud*. A more difficult factor to evaluate would be the decomposition of chlorophyll in the

phytoplankton. A large fraction of the seston suspended in lake and ocean waters consists of dead organic materials. We suspect that some of the chlorophyll degradation products present in mud may also occur in the seston. If this were the case (which only future analyses will show) then the method of Richards and Thompson (1952 (2)) for the spectrophotometric determination of chlorophylls *a*, *b*, and *c* in phytoplankton might produce erroneous and misleading results, for their method depends on the assumption that chlorophylls *a*, *b*, and *c* are the only compounds present which absorb light from 635 to 663 mμ.

Acknowledgments

We are indebted to Dr. J. S. Nicholas for permission to use the facilities of the Osborn Zoological Laboratory at Yale University during the collection period, and to the National Research Council of Canada for providing a grant to cover travel expenses. The determinations of absorption spectra were considerably hastened by the use of a Beckman DK-2 recording spectrophotometer, purchased through a grant from the Carnegie Corporation of New York.

References

1. Deevey, E. S., Jr. Limnological studies in Connecticut. V. A contribution to regional limnology. Am. J. Sci. **238**, 717–741 (1940).
2. Richards, F. A. and Thompson, T. G. The estimation and characterization of plankton populations by pigment analyses. II. A spectrophotometric method for the estimation of plankton pigments. J. Marine Research (Sears Foundation), **11**, 156–172 (1952).
3. Vallentyne, J. R. Sedimentary chlorophyll determination as a paleobotanical method. Can. J. Botany, **35**, 304–313 (1955).

PHOMOPSIS BLACK ROT OF CUCURBITS[1]

COLIN D. MCKEEN[2]

Abstract

A new species, *Phomopsis cucurbitae*, is described as the chief causal agent of a black rot disease of greenhouse cucumbers in Ontario. In addition to causing stem canker, the fungus occasionally causes decay of fruits. Similarities in macroscopic symptoms caused by *P. cucurbitae* and by another fungus, *Mycosphaerella melonis*, that also causes black rot of cucurbits, are pointed out. The pathogenicity of *P. cucurbitae* on cucurbits and tomato is discussed.

Introduction

The disease was first observed in the summer of 1946 when it was seen affecting a few plants in a glasshouse crop of cucumbers (*Cucumis sativus* L.) at Leamington, Ontario. Since that time the disease has been found every year in a relatively small number of cucumber crops grown under glass in southern Ontario, but only in 1950 and in 1954 was its incidence sufficiently high for it to be regarded as a disease of importance. In those 2 years serious losses occurred in single large greenhouses. On these occasions the disease appeared in May soon after harvesting began. Considerable stem girdling caused death of many vines or a major portion of many vines before the cropping season ended 2 months later.

Twice the disease has been observed affecting cucurbits in the field; once it caused a rot of fruits of watermelon (*Citrullus vulgaris* L.) in field plots at the Harrow laboratory, and on another occasion it caused a stem canker of muskmelon (*Cucumis melo* L.) that killed the vine.

For 4 years *Phomopsis* black rot was mistaken for the black rot disease of cucurbits caused by *Mycosphaerella melonis* (Pass) Chiu & Walker, because of a striking similarity in the general symptomatology of the two diseases. However, among other distinguishing features, the consistent absence of an associated perfect state of the black rot fungus found on glasshouse crops led the writer in 1949 to a careful microscopic study of the fungus. These investigations soon revealed that the fungus commonly found in Ontario was distinct from the cucurbit black rot fungus described by previous investigators (5, 6, 7, 14, 16). Although the occurrence of the *Phomopsis* was recorded in a paper read in 1951 (11), in the present paper the fungus is described and the results of pathogenicity studies are reported.

Symptomatology

On the cucumber plant, the host on which the disease has been observed most frequently, the disease appears as cankers or lesions on the stems, as a leaf and petiole rot, as a blight of the inflorescence, and as a fruit rot.

[1]Manuscript received August 16, 1956.
Contribution No. 1561 from the Botany and Plant Pathology Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario.
[2]Science Service Laboratory, Harrow, Ontario.

Infections are frequently observed first on senescent and moribund tendrils, fruit peduncles, petioles, and suckers that arise at stem nodes. These infections usually spread into the adjoining stem tissue where they appear at first as water-soaked, oily-green areas (Fig. 1). Amber-colored gummy exudations (Fig. 2) accompany nodal infections. The lesion spreads upwards and downwards from the node, at first involving only epidermal and cortical tissue. Later, the fibrovascular tissues are penetrated by the fungus mycelium and after the stem is entirely girdled, the portion of the vine above the lesion wilts and dies. The rate of spread of the lesion depends on the succulence of invaded tissue which, in turn, is affected by cultural conditions. Some lesions may involve only a few centimeters of internodal tissue, whereas others may extend along the stem for a distance of six or seven internodes. Lesions occasionally occur separately at one or more nodes on the same stem.

Black pycnidia first appear in the oldest part of the lesion, and subsequently develop on the affected tissue in more or less longitudinal rows (Fig. 3) in the internode a few centimeters behind the advancing margin of the lesion. Pycnidia that are embedded beneath the epidermis of the host at first appear as rather inconspicuous pin-point black dots. As the pycnidia mature and become erumpent, the affected tissue becomes paler in color until eventually it becomes blanched and somewhat separated into strips. At this stage the black pycnidia are conspicuous against the light-colored drying substrate.

By way of the inflorescence the fungus occasionally initiates an infection of fruits (Fig. 4) where the mycelium rapidly advances through the pulp tissue of the developing green fruit. Ultimately, the rind of the fruit becomes studded with black pycnidia. The underlying infected tissue becomes soft and water-soaked. Finally the fruit shrinks and becomes mummified. The infected decaying fruit emits a lemonlike odor.

Morphology and Taxonomy of the Fungus

The causal fungus produces on agar a medium-heavy, cottony, aerial mycelium that is branched and irregularly septate. Many of the cells of the older mycelium are barrel-shaped. The fungus grows well on a number of agar media. On PDA the diameter of the colony increases by approximately 2 cm. per day at room temperature. On this medium the mycelium is at first white, but after 5 to 7 days it begins to impart a color to the agar. Depending to some extent upon the isolate, but also upon the acidity of the medium, the color ranges from salmon pink to pale yellow to olive green to violet to brown, and ultimately in old cultures all these colors develop blackish tones. Although stromatic masses are not formed either in culture or in the host, blackish islands are occasionally observed on infected cucumber stems.

At room temperature about 8 to 10 days after a plate of PDA is inoculated, pycnidia begin to form in the oldest part of the colony. Five or six days later amber-colored droplets (Fig. 8) containing pycnidiospores form at the ostioles of the oldest pycnidia.

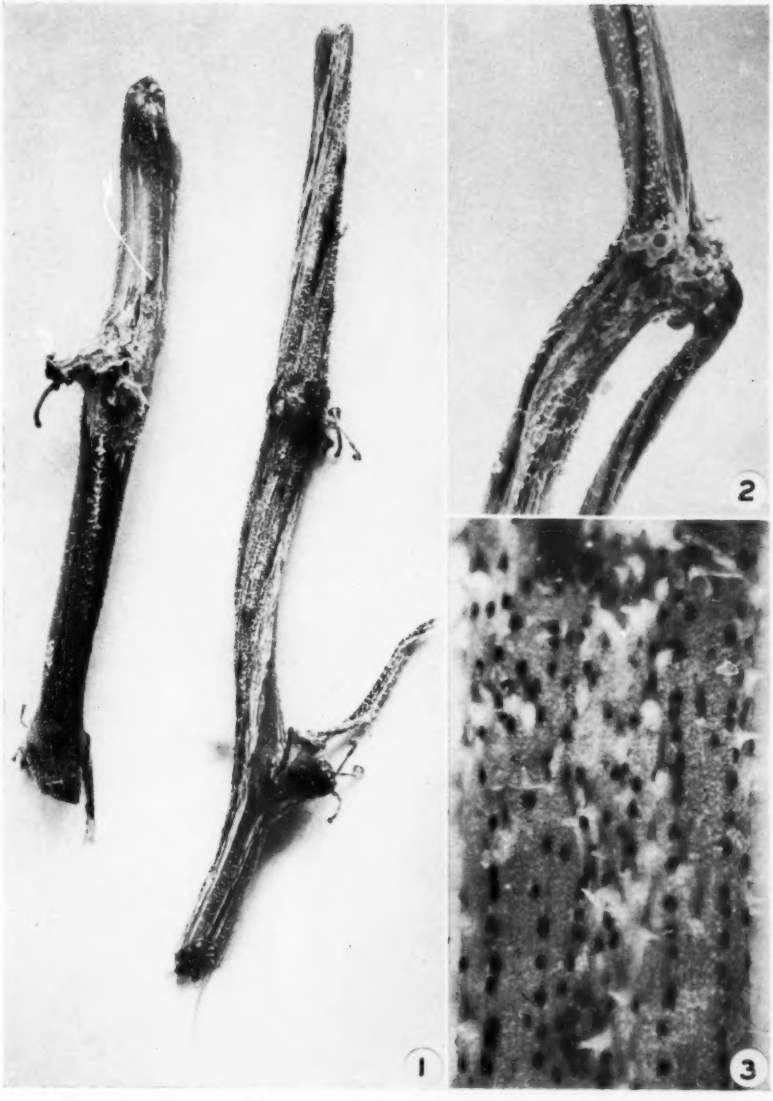
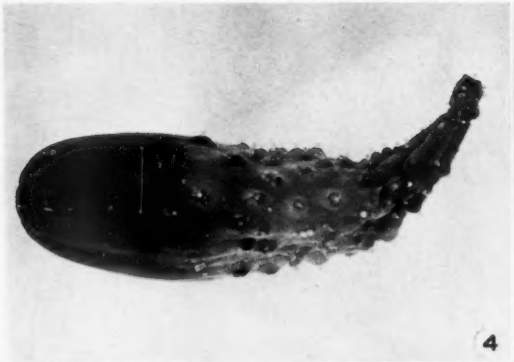
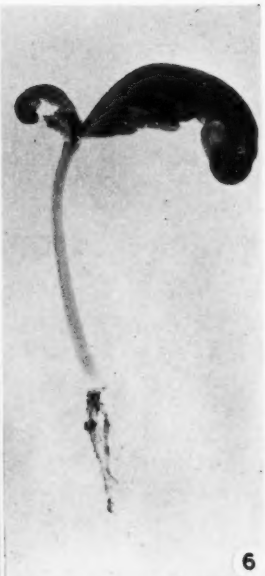
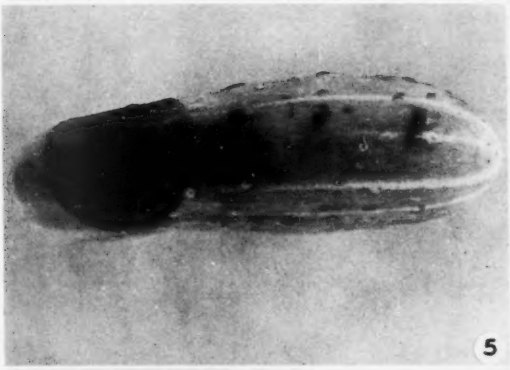


FIG. 1. *Phomopsis* lesions on cucumber stems developing from nodal infections.
FIG. 2. Gummy exudations appear soon after healthy nodal tissue is invaded.
FIG. 3. Pycnidia arranged in more or less longitudinal rows along the stem internode. ×15







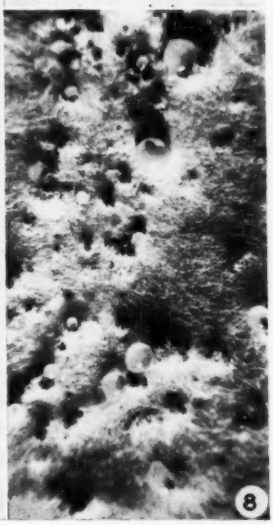


FIG. 4. Cucumber fruit infected at blossom end with *Phomopsis*.
FIG. 5. Mature green cucumber fruit inoculated through scalpel wounds.
FIG. 6. Lesions on cotyledons of squash.
FIG. 7. Rind of watermelon fruit studded with pycnidia. Pulp tissue is collapsing.
FIG. 8. Droplets containing pycnidiospores forming around ostioles of pycnidia produced in culture. ×20

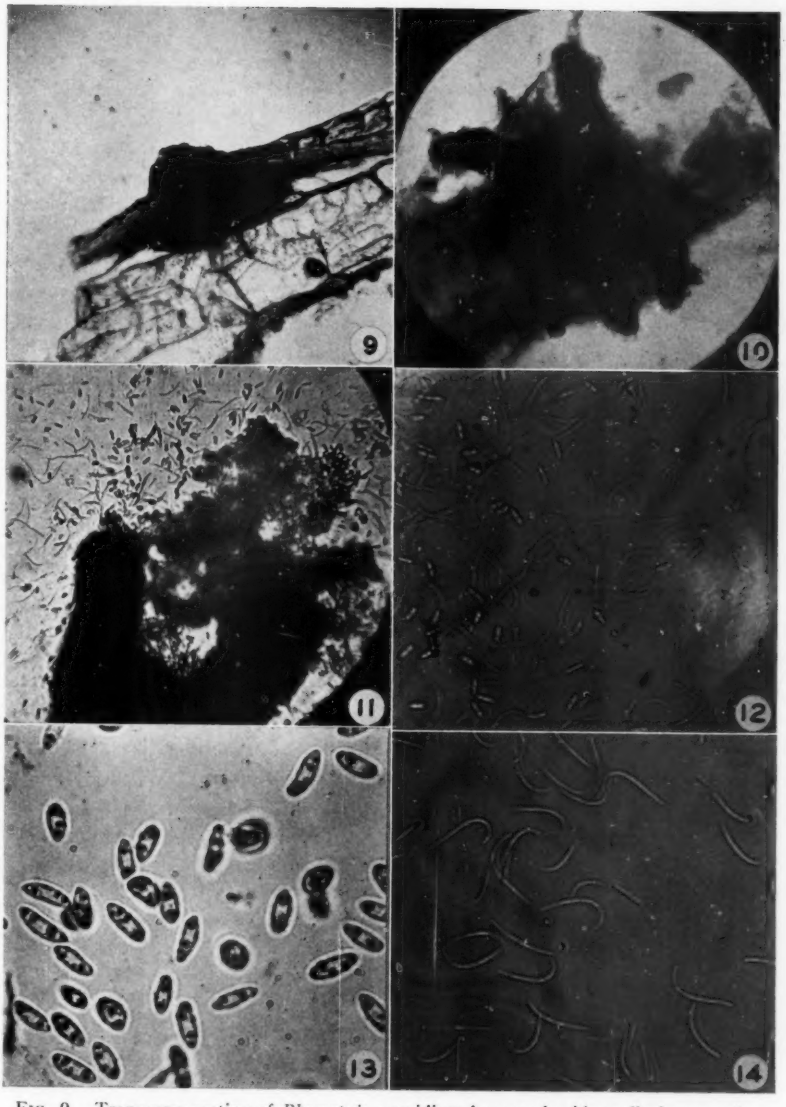


FIG. 9. Transverse section of *Phomopsis* pycnidium borne subepidermally in cucumber stem. ×100
FIG. 10. Multiostiolate pycnidium produced on PDA. ×70
FIG. 11. Crushed pycnidium exuding pycnidiospores of both alpha and beta (stylospores) types. ×200
FIG. 12. A mixture of alpha and beta spores. ×300.
FIG. 13. Alpha spores. ×1000
FIG. 14. Beta spores or stylospores. ×550

On inoculated, steamed cucumber stems a floccose mycelial growth covers the greater portion of the stem, and within 7 to 9 days at room temperature pycnidia begin to appear and develop abundantly on the area showing the sparsest aerial mycelium. The pycnidia are subglobose, flattened, with a short beak.

On naturally infected stems pycnidia develop in the subepidermal layer among thin-walled cells, and the developing pycnidium is conspicuously compressed to form a lenticular, boat-shaped structure (Fig. 9). A short beak forms which ruptures the epidermis.

On culture media pycnidia often lack a definite shape and contain more than one chamber. These pycnidia are often multiostiolate (Fig. 10).

The wall of the pycnidium is composed of compact, dark-brown hyphae. From the innermost layer of cells hyaline conidiophores arise.

Pycnidiospores are of two kinds, namely alpha spores and beta spores (Figs. 11 and 12). Alpha spores (Fig. 13) are continuous, hyaline, and contain from two to five and occasionally as many as eight oil globules. These spores are fusiform, straight, or curved. One or both ends may be rounded, or one end may be noticeably pointed, or even drawn out to a long tapering point. These spores range in length from 6.8 to 14.5 μ with an average of 10.1 μ, and in width from 2.8 to 4.2 μ with an average of 3.1 μ.

In natural infections alpha spores predominate and often to the complete absence of the beta spores. In cultures, however, the beta spore is frequently found; with some isolates that have been in culture for several months beta spores predominate and in some pycnidia they predominate to the virtual exclusion of alpha spores. Beta spores (Fig. 14) are long, slender, hyaline, curved or hooked, 14 to 25 μ long, with an average of 19.5 μ in length and 1.25 μ in width.

When mature pycnidia are compressed in a drop of water on a slide beneath a cover slip, pycnidiospores exude in a cirrus. Spores from these cirri disperse more rapidly than the pycnidiospores of *Mycosphaerella melonis*.

Pycnidia show considerable variability in character and size depending largely on the substrate on which they are borne, but also to some extent on the isolate. On naturally infected cucumber stems mature pycnidia ranged from 140 μ to 325 μ with an average of 225 μ at the least diameter and ranged from 175 to 400 μ with an average of 300 μ at the greatest diameter. One isolate obtained from watermelon consistently produced considerably larger pycnidia than any other isolate both in natural infections and in culture. On steamed cucumber stems the average diameter of 50 pycnidia of the watermelon isolate as compared to that of a similar number of the cucumber isolate was 461 μ: 288 μ, respectively, and some of the largest of the former ranged up to 700 μ in diameter.

On steamed soybean stems the ostioles of the pycnidia were located at the ends of prominent necks which were 100 μ long by 70 μ wide. Necks were not prominent on steamed cucumber stems or other artificial substrate.

The perfect state of the fungus has not been found. Various methods of overwintering naturally infected stems have failed to produce a sexual or perfect state. However, a fungus that produced perithecia on moribund tissues of cucumber plants has been observed on lesions caused by the pycnidial fungus, as reported earlier by the writer (11). Nevertheless, all attempts to demonstrate by various means, including the pairing of numerous isolates, have failed to show that the pycnidia and the perithecia are different states in the life history of the same fungus. From the evidence at hand the perithecial fungus is considered to be a distinct saprophytic fungus on cucumber. Further studies of this fungus are being made.

On the basis of the characteristics of the pycnidium and on the presence of alpha and beta spores the fungus is a species of the form genus *Phomopsis*. Inasmuch as the fungus has not been previously described or reported on cucurbits, it is described here as new, and the following name is proposed: *Phomopsis* ***cucurbitae*** sp. nov.

Pycnidia subepidermalia erumpescentia, subglobosa vel cum in caulibus lenticularia, ab atrobrunneis nigra, breviter rostrata, 140–400 μ diam., saepius unilocularia, ostiolata; conidia numerosa et generum duarum sunt; aliae, sive alpha, sporae hyalinae, uniloculares, continentes 2–5 (−8) globularum olei, fusiformes, rectae vel paullum asymmetricae, ambitu rotundatae vel ad alium apicem rotundatae alio acutae, 6.8–14.5 × 2.8–4.2 μ; aliae, sive beta, hyalinae, filiformes, uniloculares falcatae vel hamatae, 14–25 × 1.15–1.35 μ.

Habitat in caulibus, ramis, petiolis, claviculis, fructuum pedunculis fructibusque Cucurbitacearum.

Type locality: Leamington, Ontario.
Type specimen: DAOM 41000 has been deposited in the Mycological Herbarium, Ottawa, Ontario.

Pathogenicity Tests

Many tests of the pathogenicity of *Phomopsis cucurbitae* were carried out in laboratory and in greenhouse studies.

Inoculations

The conditions conducive to infection were determined by spraying healthy young, middle-aged, and old cucumber plants in the greenhouse with aqueous suspensions of pycnidiospores. Subsequently, the atmosphere was kept moist for 48 hr.

Infection did not occur when young plants were inoculated by this method. Without exception, the highest percentage of infections developed in mature, yet commercially productive, plants. Under greenhouse conditions conducive to good vegetative growth the leaves fill in between the rows and consequently, as the season advances, organs on the lower portion of cucumber stems become chlorotic and die progressively from the base of the plant up-

ward. These organs were observed to serve as primary loci of infection. The maintenance of a humid atmosphere was found to be essential for infection. Moreover, the dependence on a high state of succulence of the susceptible tissue for the establishment of infection was also demonstrated. Furthermore, the degree of succulence of such tissue determined to a great extent the rate of spread of the lesion, and consequently the abundance of pycnidia formed later thereon.

Cucumber, muskmelon, and watermelon vines were also readily inoculated by using the toothpick method (9). The tip of the toothpick upon which the fungus was growing was inserted into the stem at the node through adjoining moribund, succulent plant tissue. The exposed ends of the toothpicks were covered with vaseline to prevent desiccation. Frequently, within 3 weeks, lesions extending 2 to 4 in. above and below the point of inoculation developed. Similar inoculations on buttercup squash (*Cucurbita maxima* L.) yielded negative results.

Cucumber and watermelon fruits have been inoculated by making a small incision with the scalpel through the rind into the underlying pulp to a depth of approximately $\frac{1}{4}$ in. A small amount of agar on which the fungus was growing was inserted into the wound before the surface of the wound was sealed with a piece of cellulose tape. Decay originating at the wound developed more quickly in mature watermelon fruits (Fig. 7) than in mature green cucumber fruits. In the latter, infection (Fig. 5) did not occur until the fruit showed evidence of yellowing of the rind. Subsequently, the tissue was readily permeated by the fungus mycelium giving rise to a tan-colored lesion that gradually darkened. Occasionally, inoculations have remained dormant for 2 weeks or longer until the cucumber fruit ripened sufficiently to permit fungus invasion.

Studies as to the Seed- and Soil-borne Nature of Phomopsis in Cucurbits

Consideration of the possibility of seedling infection from infected seed or soil led to a series of experiments that involved the artificial infestation of soils. Sterilized rye grain in Erlenmeyer flasks was inoculated with cultures of *Phomopsis*. When the fungus had completely permeated the rye medium this inoculum was thoroughly incorporated into steamed soil at the rate of 4% by volume. A layer of infested soil 2 in. deep was placed over ordinary steamed soil in each 5-in. pot. Twelve seeds of cucumber, muskmelon, watermelon, or squash were planted separately at a depth of $\frac{3}{4}$ in. In these tests the pathogenicity of *Mycosphaerella melonis** was compared with isolates of *Phomopsis* obtained from cucumber and watermelon. Three separate greenhouse tests which were adequately randomized and replicated were conducted during the months of April, May, and September of 1952. During the course of these experiments greenhouse temperatures ranged from a low of 60° F. at night to a high of 95° F. in the daytime.

*A culture of *M. melonis* was obtained from Professor J. C. Walker, Madison, Wisconsin.

Neither *Phomopsis* nor *M. melonis* caused pre-emergence mortality of any of the cucurbits. Both fungi caused tan- to orange-colored lesions on one or both of the cotyledons in as many as 5% of the emerging seedlings of the aforementioned hosts (Fig. 6). Within 24 to 48 hours of the emergence of the cotyledons from the soil, lesion development was arrested. Within a month after the inoculated plants had made much growth, a high percentage of the arrested lesions resumed activity, caused death of the affected cotyledons, and on the adjoining hypocotyl and stem produced a lesion that girdled and killed the plant. On squash, but not on the other three cucurbit hosts, neither *Phomopsis* nor *Mycosphaerella* caused cankers on the hypocotyl adjacent to the infected cotyledons. By the usual cultural methods, the fungus with which the soil had been infested was recovered from the lesions on the cotyledons and hypocotyls of the affected seedlings.

Host Range Studies

Early in the present century Massee (13) confused *Didymella lycopersici*, a fungus that frequently causes a stem rot of tomatoes in the British Isles, with *Mycosphaerella melonis*, a cucurbit black rot fungus. Later Brooks and Searle (1) determined that these two fungi were morphologically distinct, and that the latter did not occur in England. More recently Moore and Moore (14) have found *M. melonis* occurring on muskmelon in England. In 1950 Hendrix reported the occurrence of *Phomopsis* on tomato in Hawaii, and recently, Ceponis and Friedman (2) reported a *Phomopsis* on tomatoes in New York. In the light of these findings it seemed important to know whether the *Phomopsis* that attacks cucurbits in Ontario will also attack tomatoes. Accordingly, by means of the aforementioned toothpick method mature tomato plants grown in the greenhouse were inoculated with *Phomopsis* isolated from cucumbers and watermelons. Care was taken to inoculate stems through senescent and dead tissue. In contrast with similar inoculations on cucumber, watermelon, and muskmelon, those on tomato stems failed to produce infection. However, inoculations of firm red tomato fruits produced obvious lesions in 72 hr. and this tissue was colonized almost as readily as that of watermelon fruits. *M. melonis* also readily invaded red tomato fruits. Neither fungus produced pycnidia on the surface of the tomato as they did on cucumber and watermelon. There is a distinct possibility that the *Phomopsis* sp. reported as being pathogenic to tomatoes by Hendrix and by Ceponis and Friedman may be the same species of *Phomopsis* that attacks cucurbits in Ontario.

Control

Spraying infected plants with fungicides such as ferbam, wettable sulphur, and fixed coppers failed to check the spread of the disease.

Good ventilation resulting in lowered humidity has been found beneficial in checking the spread of the disease because rapid desiccation of senescent and dying plant parts such as petioles, tendrils, fruit peduncles, and blossoms provides unsatisfactory infection courts.

Discussion

Since 1949, when *Phomopsis* was found to be a causative agent of black rot of cucurbits in Ontario, a careful examination of the fungi causing black rot on cucurbits grown in greenhouse and in field crops has been made. *Mycosphaerella melonis* has been isolated only once in 7 years, whereas *Phomopsis* has been found affecting one or two glasshouse cucumber crops every year during that period. Because the striking similarity in macroscopic symptoms and pathogenicity of these two distinct fungi may readily lead to misidentification, it was at first thought that earlier workers might have misidentified these fungi. A careful scrutiny of the descriptions of black rot fungi studied by Chester (3, 4) at the Delaware Experimental Station in 1891–93 and also those of Massee (13), who reported only an imperfect state of the fungus, makes it appear somewhat unlikely that *Phomopsis* was encountered by them.

The mode of transmission or the source of inoculum of *Phomopsis* for cucurbits in southwestern Ontario remains obscure. It may be seed-borne like *Mycosphaerella* and *Colletotrichum lagenarium* as reported by Rankin (15). However, in the initiation of an outbreak of *Phomopsis* stem canker in isolated glasshouse cucumber crops, the occurrence of lesions on the uppermost internodes of affected plants in the complete absence of lesions on the cotyledonary and lower internodes would suggest the introduction of inoculum by a channel other than through infected seed.

Phomopsis cucurbitae is an example of a fungus that depends on a period of growth as a saprophyte before it invades healthy tissue. Its pathogenic capabilities are similar to those of *Botrytis cinerea*, and *Trichothecium roseum* as reported by the writer (10, 12). These three fungi occasionally become important pathogens in cucumber crops grown under glass in Ontario, yet they seldom appear in the field.

Acknowledgments

The writer wishes to thank Dr. J. Walton Groves, Botany and Plant Pathology Division, Ottawa, Ontario, for much appreciated assistance with the mycology and naming of the fungus, and for helpful criticism of the manuscript. Grateful acknowledgment is tendered to Dr. Bernard Bovin, Botany and Plant Pathology Division, Ottawa, for preparation of the Latin diagnosis. The writer also wishes to thank Dr. L. W. Koch, Officer-in-Charge of this laboratory, for bringing this fungus to my attention in 1946, and also for reading the manuscript.

References

1. BROOKS, F. T. and SEARLE, G. O. An investigation of some tomato diseases. Trans. Brit. Mycol. Soc. **7**, 173–197 (1921).
2. CEPONIS, M. J. and FRIEDMAN, B. A. The occurrence of *Phomopsis* on tomatoes in New York. Plant Disease Reptr. **39**, No. 12, 989–990 (1955).
3. CHESTER, F. D. Notes on three new or noteworthy diseases of plants. Bull. Torrey Botan. Club, **18**, 371–374 (1891).

4. CHESTER, F. D. Report of the mycologist. Delaware Agr. Expt. Sta. Ann. Rept. **5**, 53–80 (1893).

5. CHIU, W. F. and WALKER, J. C. Morphology and variability of the cucurbit black rot fungus. J. Agr. Research, **78**, 81–102 (1949).

6. CHIU, W. F. and WALKER, J. C. Physiology and pathogenicity of the cucurbit black rot fungus. J. Agr. Research, **78**, 589–615 (1949).

7. GROSSENBACHER, J. G. A *Mycosphaerella* wilt of melons. N.Y. Agr. Expt. Sta. Tech. Bull. **9** (1909).

8. HENDRIX, J. W. A *Phomopsis* disease of tomatoes in Hawaii. (Abstr.) Phytopathology, **40**, 966 (1950).

9. HILDEBRAND, A. A. An elaboration of the toothpick method of inoculating plants. Can. J. Agr. Sci. **33**, 506–507 (1953).

10. MCKEEN, C. D. Observations on the occurrence and control of grey mould on greenhouse cucumbers. Sci. Agr. **32**, 670–676 (1952).

11. MCKEEN, C. D. An aberrant strain of the black rot fungus causing fruit and stem rot of greenhouse cucumbers in Ontario. (Abstr.) Phytopathology, **42**, 16 (1952).

12. MCKEEN, C. D. *Trichothecium* foliage rot of greenhouse cucumbers. Can. J. Agr. Sci. **34**, 469–472 (1954).

13. MASSEE, G. Cucumber and tomato canker (*Mycosphaerella citrullina*, Grossenb.). Kew Roy. Botan. Gardens Bull. Misc. Inform. **7**, 292–293 (1909).

14. MOORE, W. C. and MOORE, J. F. Stem rot of melon (*Mycosphaerella citrullina*). Trans. Brit. Mycol. Soc. **32**, 277–279 (1946).

15. RANKIN, H. W. Effectiveness of seed treatment for controlling anthracnose and gummy-stem blight of watermelon. Phytopathology, **44**, 675–680 (1954).

16. WIANT, J. S. *Mycosphaerella* black rot of cucurbits. J. Agr. Research, **71**, 193–213 (1945).

DEVELOPMENTAL STUDIES OF ELODEA CANADENSIS MICHX.

II. EXPERIMENTAL STUDIES ON MORPHOLOGICAL EFFECTS OF DARKNESS[1]

HUGH M. DALE

Abstract

A description of the morphological characteristics of sprigs grown in the dark is given. Among the factors that may be responsible for the pattern of growth there is the absence of direct light stimulus, the effect of the sugar solution, the lack of auxins, and the oxygen factor. Experimental evidence suggests that the lack of light per se has little effect upon the form of *Elodea*. Indole-3-acetic acid in the concentrations used indicate only a stimulation of root production. The effect of the sugar solution appears quite noticeable. Gaseous exchange in cotton-plugged flasks is of significance particularly in dim light. Also, in plants grown with and without added oxygen, in darkness and light, it has been established that the relative amount of oxygen is a major factor influencing not only the size of cells in the leaves and internodes but also the type of air space produced. Finally, changes of gas pressure within the intercellular spaces do enlarge these spaces so that lacunae can be made to occur even in the apical cone.

Considerable attention has been devoted to the causal morphology of lysigenous lacunae which result from the death and disintegration of cells (6, 8, 14, 20, 22, 28, 29, 32). The development of other type of air spaces, which are produced schizogenetically because of the splitting apart of cell walls and later the division of cells which surround the air spaces in certain planes, has been traced in *Elodea densa* (17) and in *Elodea canadensis* (12). However, with the exception of Allsopp's observations on *Marsilea* (1,2), little attention has been paid to the causal features of development of this type.

As a general rule parts of plants growing without light develop lysigenous air spaces, whereas the illuminated parts frequently contain schizogenous spaces. Thus attempts were made to grow *Elodea* in the darkness to see if the schizogenetic type was inhibited. As a food, a supply of 2% sucrose was added to dilute Pfeffer's solution (one-fifth strength) in which micronutrients in the form of Hoagland's AZ solution (16) were incorporated. This was sterilized for 20 minutes in an autoclave at 15 lb. pressure. The usual techniques for growing plants under aseptic conditions could not be followed. Normally a seed with fairly resistant coat is surface sterilized and then grown in a sterile medium. With *Elodea* this is not practical, as seed is not available, so that attempts at surface sterilizing whole shoots were made. *Elodea* is a plant with very delicate leaves and internodes, therefore in most tests, the surface sterilizing agent was either strong enough to kill the plant or too weak to destroy all the contaminants. As a result, only in a small fraction of cases was contamination eliminated. Short term experiments for a week or 10 days yielded useful data, but in the hundreds of attempts, only a few shoots were successfully grown for longer periods. In the most successful experiment, the following procedure was followed. In October 1952, a group of short apices

[1]Manuscript received October 18, 1956.
Contribution from the Department of Botany, University of Toronto, Toronto, Ontario. Part of a thesis presented at the University of Toronto in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

from plants that had been growing vigorously in Grenadier Pond all summer were cut to about $\frac{3}{4}$ in. and left for 2 hours in saturated bromine water diluted to one part in five hundred, with a drop of "Tween 20", a water soluble, non-ionic surface active agent, added. They were then lifted with a nickel loop which had been sterilized by being heated to white heat, placed in 60 ml. Erlenmeyer flasks containing 40 ml. of the nutrient solution, and kept at constant temperature of 25° C. with an illumination of approximately 150 ft-c. outside the flasks for 16 hours per day. The tips were moved to fresh flasks every second day with a sterile loop until it was thought that all the contaminants had germinated and been eliminated. In the particular group of October 1952, 10 tips seemed to be free from infection and were transferred to 250 ml. flasks which were plugged with non-absorbent cotton, wrapped in new aluminum foil, and covered with a loose cap of aluminum foil. The wrapped flasks were then placed in a stone crock, capped with the foil. The crock was placed in a 25° C. constant temperature tank in a dimly lit tank room and left undisturbed until the middle of December when four of the flasks were still uncontaminated and the tips showed considerable growth. Attempts to follow this same method, using stock material from the greenhouse aquaria, were less successful and it was not possible to keep tips growing without contamination for a comparable length of time. (The contamination may be internal in many cases, for in examining sections of normal plants, one occasionally encounters threads of mycelium growing through the cells of the leaf and stem.)

When the plants that had been grown for 2 months in complete darkness were examined, it was found that they had not grown as vigorously as those in a dim light. As one would expect, they were white rather than green, and their appearance was one of delicacy. However, many of the outward appearances of etiolation that have been so frequently described in dicotyledons (19, 23, etc.) are absent in *Elodea*, as is the enlargement of internodes, leaf blade, and articulation of leaf that might be expected to occur in this monocotyledon (27, etc.). The leaves are smaller than those of plants grown in the light, with fully expanded leaves averaging only 3.3 × 1.5 mm. compared with 10.0 × 1.6 mm. for those developed at 25° C. with medium light for a photoperiod of 16 hours when the plant was rooted in soil. The internodes are also smaller, with mature ones measuring only 2.4 mm. in length and 0.5 mm. in diameter (compared with 10.3 mm. and 0.6 mm. from sprigs grown under the aforementioned conditions in the light). When sections were examined it was found that the small size was due to a lack of cell enlargement. This is in contrast to many plants in which large-celled watery tissue results from growth in the darkness. Only 40 nodes of measureable length were laid down by each sprig in the 2 months without light (about half the number encountered in sprigs grown in the light at that temperature). Eight branches were initiated and no roots emerged.

The nodes that grew as a result of the elongation of the main axis had three leaves whereas nodes on branches which grew from their initiation in the

darkness had only two leaves per whorl. On examining the branch apices it was found that they were only three-fourths the size of light-grown apices. Using Stant's criterion for size (31), it was found that a typical one measured only 94 mm. from the tip to the base of the first leaf primordium and only 67 mm. wide. The smaller size was due to fewer cells. This demonstrates the fact that whenever a narrowed apex in *Elodea* is due to a reduction in cell number, fewer leaves per whorl are initiated. This has also been observed in *Elodea* grown under a variety of conditions. Further, it has been pointed out that under natural conditions the basal two to four nodes of each branch support only two leaf appendages, whereas closer to the tip when the apical cone has enlarged, whorls of three leaves are general (12). *Elodea densa* has an apical cone of larger size (31) and in this species four leaves per node are initiated.

Immediately below the initial cells, other differences are noticeable in these apices. The cylinder of meristematic tissue, above the first leaf primordia, is short. The cells of this region, the rib meristem, are less orderly in appearance. In Figs. 1 and 3 of the first article in this series (12), illustrating normal tips, the rib meristems show as regular rows of evenly sized cells. Comparable sections from tips grown in the dark suggest that cell division is not restricted to the anticlinal type, although that type is the most prevalent. There is no sign of chinks between these cells, although the lumen appears rounded, giving the appearance of young nodal cells except that the nuclei occupy a large percentage of the cell volume.

Growth is slow in these plants, although the carbohydrate supplied by the substrate is so abundant that starch is prominent in the sections.

The leaf whorls are initiated in the normal manner, as are branch buds, the latter remaining narrow and producing only two leaves per whorl. As in the normal development the slits that appear in the cortical tissue of the stem at the time of leaf initiation are produced.

Cells forming the start of internodes appear to be produced from the division of cells at the base of each leaf insertion disk. The slits at the corners of the cells enlarge and coalesce to form the start of the elaborate internodal cavities. Then, in some cases, the nodal cells divide longitudinally, as in the shoots grown in the light,* enlarging the cavity and producing a regular schizogenetic lacuna. In other cases the horizontal growth and division of the nodal cells fail to outpace similar processes in the internode so that internodes are initiated which are solid, except for long chinks at the corners of the cells. This condition has been traced through six adjacent nodes, indicating that this pattern was well established in one of the side branches. Other internodes indicated this same tendency. Fig. 1 illustrates this. The top half of this section is devoid of lacunae although the chinks at the corners of the cells are easily discerned. In the bottom half of the figure schizogenous air spaces are illustrated although many of the cells are partially collapsed. It will be noted that the width of the internode is greatly reduced and contains

*Seen in Fig. 10 (12).

a single circle of large lacunae as compared with two circles in the wider, normal internode.* When the lacunae pattern has been established, cell division lengthens the internodes but at maturity they are still short owing to a lack of cellular elongation. Presumably carbohydrate deficiency cannot have been restricting, as in all sections of the plant starch is abundant.

In many sections no further development of internodal air spaces occurs, but a widening of the internode may involve the loss of contact between adjacent cells. This results in a somewhat torn appearance of sections. In Fig. 3 a large opening occurs at the top of the section. Large irregular lacunae of this type were frequently encountered in this material and appear to be produced by a splitting apart of cells at the middle lamella. Another type of enlargement occurs when cortical cells appear to collapse and die. Fig. 2 and the bottom half of Fig. 1 illustrate the enlarged holes produced when the cortical cells collapse. Frequently the pattern of formation can be traced. The cells swell, often with plenty of evidence of starch, and later these cortical cells collapse leaving the lacunae.

The pattern of the formation of some of these cavities is similar to that seen by McPherson (20) in the roots of *Zea mays*, where lysigenous spaces form. However it should be pointed out that many of these spaces are formed in *Elodea* in the presence of starch grains (while the plant was growing in a sucrose solution). Fig. 4 illustrates a cross section of an internode in which the cells are collapsing to produce lysigenous spaces. The abundant starch grains appear as light dots. Fig. 6 illustrates a small segment of the section illustrated in Fig. 4. It is photographed with an oil immersion objective. It is clear that starch is present even in the cells that have lost their turgor. McPherson (20) believed that the death of the cells in the roots of *Zea mays* occurred with starvation, the anaerobic respiration using up the carbohydrate, then the protoplasm itself. Aeration of the liquid in which he grew the roots presumably produced the more efficient aerobic respiration and the spaces did not form.

*See Fig. 14 (12).

FIGS. 1–8. Photomicrographs of *Elodea canadensis* fixed in formalin–aceto–alcohol, dehydrated, and embedded in paraffin by the tertiary – butyl alcohol method; microtomed; and stained with Delafield's haematoxylin.

FIG. 1. Transverse section of branch shoot ×65; 2% sucrose without light; upper segment devoid of lacunae; lower section with schizogenous spaces, some cortical cells collapsing. Leaf section devoid of vascular tissue.

FIG. 2. As FIG. 1. Lysigenetic spaces developed as a result of cortical cell collapse. Vascular tissue only slightly developed in leaf section.

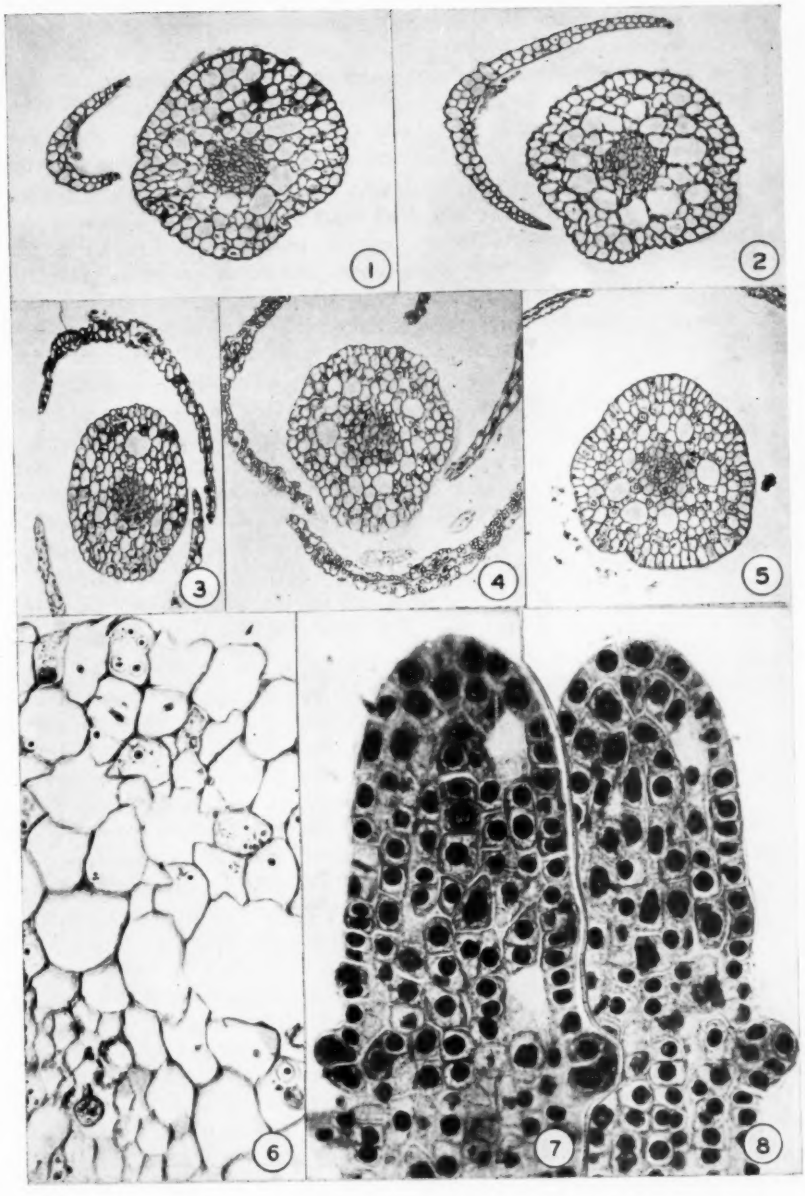
FIG. 3. As FIG. 1. Large air space at top of internode segment produced by loss of contact of cell walls.

FIG. 4. As Fig. 1. Cortical cells in condition of incipient collapse. Many starch grains prominent.

FIG. 5. Transverse section of shoot internode ×65; 5% sucrose dim light. Schizogenous lacunae in all but segment at lower right.

FIG. 6. Transverse section of shoot ×500, enlarged part of FIG. 4.

FIGS. 7 and 8. Longitudinal section of shoot apex ×425, medium light; 16 hours per day illumination with alternating pressure. Large spaces in apical cone.


1
2
3
4
5
6
7
8

Dr. Sifton (private communication) has said that in the disease of bitter pit in apple, lysigenous spaces form and cells full of starch disintegrate to produce the cavities. This is the condition which occurs in *Elodea*. If starvation is the immediate cause, the only suggestion that presents itself is that there is a lack of the conditions necessary either for digestion of starch or for anaerobic respiration. At any rate it is clear that sugar is being absorbed from the external solution and condensed into starch.

In this dark-grown material nourished with 2% sucrose, a variety of air space formation is found in the same flask and on the connected branches of the same shoot. Schizogenetic lacunae are initiated as in shoots grown in light. Their growth is always restricted so they are small at maturity. These cavities may enlarge lysigenously as cells collapse. In other branches no lacunae are initiated but the cortex appears as a solid tissue. Cavities in these internodes may develop owing to loosening of cell connections or they may develop lysigenously as cells collapse.

The central vascular stele appears to be differentiated in the same way as in other experimental material. However the small strands in the outer cortex are reduced in number, to four rather than six, this reduction being correlated with the presence of only a pair of leaves at each node. The midrib of each leaf is short. The development is acropetal, but the strand migrates only part of the way into the leaf, so that the upper part of each leaf is devoid of vascular tissue or procambium. Figs. 1 and 2 show this lack.

Sugar Solution with Light

A check experiment was planned in the hope of ascertaining whether some or all of the modifications noted when the plant was grown in darkness could be due to the presence of the sucrose in the solution. This was thought to be desirable particularly in the light of Allsopp's work (2, 3). He reports that although at lower concentration of sugar, as used here (2%), little or no modification takes place in air space tissue of *Marsilea*, the lacunae are reduced at higher concentrations (4 to 5%). Sprigs similar to those used in the experiments in darkness were grown in 200 ml. Erlenmeyer flasks containing the same 2% sucrose solution. The flasks were plugged with non-absorbent cotton and were immersed to the top of the liquid in a tank of water at 25° C. They were illuminated by a 200 watt floodlight from above. The cultures were not sterile and to minimize the effect of contamination the material was moved to a fresh flask three times a week. After 45 days the material was examined, and it was found that leaves and internodes were all short. The apical cones are of similar width to those grown in light while the lengths are all below the average of those grown normally in light but much longer than those grown in the dark. It should also be mentioned that the air spaces developed early.

A second experiment was performed that was identical with the above except that the containers were only of 60 ml. capacity and 5% sucrose solution was used. This was the concentration used by Allsopp (2, 3) to produce

profound changes in the lacunae of *Marsilea*. Fig. 5 is a cross section of a mature internode of one of these sprigs. In the cortex, which is reduced in thickness, it will be seen that the schizogenous lacunae have developed regularly in most of the section but are absent in a segment at the lower right. Lysigenous spaces do not occur.

In older sections the stele is of normal size for *Elodea* but occupies a larger fraction of the section owing to the reduction of the cortex. The hole in the center, representing the xylem, is larger owing to the breakdown of two xylem cells, instead of one, and a further tearing of the surrounding cells. An increased amount of vascularization has also been noted in *Marsilea*, with higher sugar concentrations of 4 and 6% (3).

From those two experiments it is not possible to decide whether the modifications produced were due to the sucrose in the medium. This has been thought of as an osmotic effect, but it is hard to believe that there would not be a compensating shift in the osmotic conditions within the cells of *Elodea* to maintain a normal gradient between the cells and the external solution. Although the effect of the sucrose solution must be left as a major consideration, yet other factors should be considered. As the contaminating population of bacteria increased, the milkiness must have reduced the amount of light considerably; moreover the respiration of these microbes must have lowered the oxygen supply. However it can be definitely stated that the modifications resulting from growth in darkness could not be produced in light in this material solely by the use of the same solution or a somewhat more concentrated one.

Addition of Indole-3-acetic Acid

Although there was no thought of pursuing the effects of growth promoting substances, yet it was thought pertinent to discover if a deficiency of this hormone in sprigs grown in the darkness might account for any of the changes in structure. Surface sterilized material was grown simultaneously under the same conditions as in the complete darkness experiment described previously, except for the addition of indole-3-acetic acid at a concentration of 1 mg. per liter. After the two month period in darkness, there were approximately the same number of nodes and branches produced as without the auxin. Externally the greatest difference was in the number of roots that had emerged from the cortex, 11 instead of zero without the IAA. The internodes were twice as long (4.8 mm.) and wider (0.7 mm. wide compared with 0.5 mm.). The leaves were more than twice as long and were wider. The main axis had three leaves per node while the side shoots each had only two leaves, a condition identical with that of shoots grown in the dark without the IAA. When sections of the sprig grown in IAA are examined, the cells are seen to be larger in the apex and throughout the whole sprig, accounting for the larger dimensions. Within the cells starch grains are even more numerous than in those grown in the companion flasks.

When the air space development in this material is examined, a series of patterns is seen, as there was without the addition of IAA, although complete suppression of lacunae in the whole of the internode did not occur. In short, sprigs grown in the flask with IAA show the same tendency as the material grown in the dark without the auxin, but it is not expressed as strongly. This difference may be due to the addition of this substance or due to other differences in the environment such as oxygen concentration. As mentioned previously, the response of all branches in the same jar is not uniform, but varies with the time of growth. Undoubtedly the availability of oxygen to any branch is not constant and will depend, among other factors, on the mass of respiring tissue in the flask. Even differences in the tightness of the cotton plugs may be a significant factor in producing the variations found in the flasks. Myers (21) has claimed that, in using cotton plugged flasks for physiological experiments, often only gas diffusion, as determined by the characteristics of the vessel, is being studied. And the claim has been made (20) that lysigenous air space formation can be prevented by the aeration of the solution. It may safely be said that the lack of indole-3-acetic acid does not inhibit the production of air spaces. It is interesting to note that Allsopp (1) reports that, in *Marsilea drummondii*, the large cortical schizogenous air spaces were suppressed with IAA when the sporocarps were germinated in a sugar solution stiffened with agar, in small tubes plugged with cotton and capped with cellophane. They were grown either in darkness or in dim light (less than 100 ft-c.) measured outside the tubes. Further Allsopp mentions that after prolonged exposure, there appear on the roots dark swellings that are due to "irregular enlargement of the cortical cells leading to their loosening and separation" (page 172). This might very easily be interpreted as the formation of lysigenous spaces. Here too oxygen is at a premium, as in the previously described experiments.

When sprigs of *Elodea canadensis* are grown in continuous light at 20° C. with the addition of 1 mg. per liter of IAA, little difference is noticed between these and the sprigs grown under similar conditions without the auxin. The air space development seems to follow the usual pattern without suppression of the schizogenous type. The initiation of roots seems to be favored. They are often formed before the elongation of the branch bud that usually accompanies a root at a node. Generally an advanced growth of the bud occurs before the appearance of the root. Further investigations will reveal whether changes in concentration of the active substance will favor branching rather than rooting.

Addition of Oxygen

Besides the effects the lack of light may produce directly upon the morphology and upon the auxin concentration, the darkness also reduces the amount of oxygen. *Elodea* therefore was grown in darkened 200 ml. Erlenmeyer flasks containing 2% sucrose and dilute Pfeffer's solution as described. The sprigs were grown at 25° C. for 45 days. Aseptic material

was not available and to minimize the effects of contamination, it was moved to a fresh flask three times a week. The change was made in very dim light with a nickel loop sterilized by heat. Oxygen was led into the covered 4 liter jars containing the flasks of material.

When the sprigs were examined they appeared like those grown in the dark without added oxygen, although the flasks were heavily contaminated at the end of the experimental periods. Examination of the stem sections, however, revealed that although the internodal width remained small, the air spaces were all schizogenous. This suggests that the significance of light in the development of air spaces rests on the production of oxygen in photosynthesis. An adequate supply of externally applied oxygen ensures the development of the schizogenous lacunae.

Growth with Readily Available Oxygen

In the preceding section it has been established that one of the important factors in producing the form of the plant encountered in the dark, is the lack of oxygen. This is particularly true with respect to air spaces. Vertical divisions in the developing nodal tissue appear to be restricted so that at times the young internodes are a solid core of cells with small chinks at the corners. This section of the present work will deal with some experiments aimed at elucidating the effects of augmented supplies of oxygen.

1. Aerial Habitat

Attempts were made to grow *Elodea canadensis* in moist air. In spite of the fact that lacunae occur normally in aquatic as opposed to land plants (5, 7, 9, 26, 30, 36) or in land plants as a result of the flooding of the soil (18), this experimental work was done to determine if a short diffusion path for the oxygen of the air to the site of the air space production would result in an enlargement of the spaces. Devaux (13) in his investigation on the composition of gases within the single large air bubble comprising all the air spaces within the stem, leaves, and roots of an aquatic claims that for a great deal of the time, gases diffuse into and out of this bubble as if there were but a single film of water between it and the air, and as a result the composition tends to be the same as free air. Nevertheless, it must be recalled that, during strong photosynthetic activity, the plant swells, as the oxygen dissolves away slowly. The large internal bubble swells sufficiently to break at small holes in the plant's surface and streams of small bubbles are extruded. If strong photosynthesis occurs for some time, the composition of these small bubbles changes until almost pure oxygen is leaving the aquatic (34). In very restricted light, or in darkness following periods of strong light, respiration continues and replaces the oxygen with very soluble carbon dioxide. Its solution results in a reduced pressure.

Elodea plants grown in moist air to reduce the diffusion path for gases do not remain healthy long, and growth, including the enlargement of chinks into lacunae, is retarded. In a tip where measurements of the size of the

lacunae have been made, it has been found that one lacuna 0.02 × 0.02 mm. in longitudinal section occurred at 1100 microns from the apex, as compared with 900 μ in a normally grown tip. However it is not known whether these lacunae were formed before or after the tip emerged from the water, and it has not been possible to keep aerial tips growing long enough to be certain on this point. In any case, it would seem that the aquatic medium is an important factor in the production of these spaces as in the other phases of the plant's growth.

2. Dissolved Oxygen in Habitat

The next experiment was performed to see if increased oxygen tension in the water would affect the development of air spaces noticeably.

Two jars were planted with *Elodea canadensis* and placed in the 20° C. tank, with continuous light of medium intensity. Air was continuously bubbled through the jars, except that one had oxygen substituted for air for 1 hour of each 24 hours. When the shoots were examined at the end of 1 month, the internodes were 17% smaller in length and width with applied oxygen, and the leaves were reduced about 20% in length. When comparative measurements of the cells from the central portion of the leaves are examined, they show at the end of the month a decrease in size that accounts for this. The treatment of the jars was reversed and the pattern followed, with shorter cells and internodes with applied oxygen, although the differences were not as striking. Measurements of the pH were made throughout the investigation and it was found to be remarkably constant at 7.5 in each jar, doubtless buffered by the soil. It is clear that the substitution of oxygen for part of each day has shortened the cell length, and thus the internodes and leaves have become reduced. This may be interpreted in a number of ways. The oxygen stream through the jar will saturate the water with oxygen and make more oxygen available to the plant, but at the same time, it will remove dissolved carbon dioxide from the medium and restrict the duration of photosynthesis, and so the amount of gaseous oxygen within the plant. It is clear however from the behavior of plants with alternating day and night that the interruption of photosynthesis even for considerably longer periods does not restrict cell length in internodes and leaves.*

An alternative explanation is suggested by a study of hardening epidermal cells. In his investigation, Watson (35) came to the conclusion that the shape and size of epidermal cells is due to the hardening of the cuticle in an oxidation process. This hardening of cell wall as a result of oxidation of the fatty material is also involved in the failure of roots of *Elodea* to form root hairs, while roots with an unsaturated fatty coating produced root hairs in light (10, 11). This explanation is quite tenable for the reduction of all length in the present experiment in view of the fact that microchemical tests reveal the presence of fatty material on the outside walls of the epidermal cells.

*These data to be published later.

In examining herbarium specimens it will be noted that in the terminal portions of some shoots, the internodes are shortened giving a crowded appearance to the leaves. The leaf length is also restricted, producing an apparent narrowing towards the tip. This is characteristic of plants growing in shallow water with strong light. As well as the influence of the light itself on the shoot tip, the surface layer of the water, when choked with growth, circulates but little, and the temperature is often raised 8° C. or more. A third factor that may contribute to this form is the large amount of oxygen present. This gas in strong light is extruded from the aquatic vegetation and must saturate the few inches nearest the water surface.

When a detailed comparison was made of apices grown with a continuous stream of air and those grown with the addition of oxygen, it was seen that the enlargement of the larger chinks into lacunae of the dimensions of the surrounding cells occurs farther from the apex in the material with the extra oxygen supply. At maturity the size and number of the lacunae is normal in both cases.

3. Internally Augmented Oxygen Supply

The effect of increasing the internal supply of oxygen was tested by increasing the rate of photosynthesis with strong light and extra carbonate and by bubbling carbon dioxide in a stream of tiny bubbles through the jars, either continuously or for only 1 hour a day. Apices from these shoots all have chinks, chiefly in the vertical direction, that run up to the initial cells of the apex. These chinks are very small but are quite discernible in prepared sections with an oil immersion objective. They are also apparent in apices grown under the strong light of their own unshaded habitat. Both the light and the carbonate in the natural environment of the individuals examined were sufficient to produce this result, whereas apices grown under the restricted light of laboratory conditions show no evidence of these chinks.

Priestley (24, 25) mentions these chinks that occur between the meristematic cells of the shoot apex. He states that they are full of liquid and suggests that they form paths of conduction from the ends of the vascular strands to the newly formed and dividing cells of the meristem. However, if apical cones are cut from shoots growing in strong light and examined immediately, while still alive, dark lines are seen, indicating the presence of these chinks filled with gas instead of liquid. The appearance is in every way similar to that of those gas-filled chinks occurring between the cells of the leaf lamina of *Elodea*. When similar apical cones are examined on dull days, the chinks are no longer obvious. However with a high power objective, their course can be traced at the corners of the cells. It is clear that under these conditions they are filled with liquid. It is a safe assumption that during strong photosynthesis these spaces are filled with oxygen, but in darkness the soluble carbon dioxide will cause a deficit of pressure, drawing liquid into the chinks. Undoubtedly Priestley (25) observed this liquid in the apices he examined. The replacement

of the liquid by gas also explains why the tips of sprigs become buoyed up under strong photosynthetic activity. The swelling of the stem with pressure contributes only part of the extra buoyancy.

In these shoots, the further development of the air spaces seems to proceed in a manner similar to that found in other shoots. Larger air spaces occur closer to the tip in shoots with unrestricted photosynthesis and the accompanying abundance of oxygen. In short, air spaces at all stages of development occur closer to the apex, indicating that their development proceeds faster, relative to the maturation of the surrounding cells. To test whether this result is due to the increased supply of food rather than oxygen, shoots were grown without the high level of photosynthesis resulting from extra carbonate and strong light, with their basal sections inserted in a hole made in a rubber tube with a red hot needle. The tube contained oxygen at a pressure of 7 cm. of mercury for 1 week, after which it was raised to 25 cm. for 3 weeks. The object was to inject the lacunae with oxygen at higher than normal pressure. The end result as far as air spaces were concerned was identical with that produced by strong photosynthetic activity.

Growth with Alternating Pressure

Under normal conditions, water plants are subject to diurnal fluctuations of gas pressure within the cortical lacunae of their stems. Since oxygen is comparatively insoluble in water, its rate of diffusion away from the plant is comparatively low and when photosynthesis is active the pressure within the plant builds up, distending the plant until the gas overcomes the surface tension of water at some opening through which it is extruded as bubbles. If the bubbling is sustained it has been noted that the gas becomes, with time, less rich in nitrogen, until it contains as much as 90% oxygen (34) and usually gives characteristic tests for this gas. The pressure built up within the lacunae of the plant is inversely proportional to the radius of the perforations through which the gas is extruded (33). Bubbling from aquatics has been traced and it is found that it invariably occurs from holes or tears in the surface of roots, stems, or leaves (34). In *Elodea canadensis* the gas water interface, determining the maximum internal pressure, will occur at the nodal diaphragms between intact and flooded lacunae.* In *Elodea densa* the measured pressure has been found to be 15 cm. of mercury above air pressure (4). It is clear that the pressure is by no means negligible.

During the hours in the dark, oxygen is used up in respiration, and the more soluble carbon dioxide dissolves and diffuses away, reducing the pressure in the lacunae. (In *Elodea densa* a low pressure of 2 cm. is recorded (4)). This pressure change is thought to influence the form of the plant. Goebel (15) suggested that gas pressure is the immediate cause of air space production, but made no explanation of how pressure might produce the effect. With this in mind, it was thought that an investigation into this thesis might be profitable.

*Illustrated in Fig. 18 in the previous article in this series (12).

A siphon arrangement was set up so that a pressure of 10 ft. of water might be built up and collapsed approximately once every 5 minutes and applied to a 4 liter jar containing *Elodea* sprigs. The experiment was run for a month, with the pressure alternating for 16 hours a day. During this time, a light from a 150 watt floodlight was directly on the jar, 1 meter distant (approximately 200 ft-c.). The pressure built up slowly during most of the 5 minute period, whereas it was released more quickly (in about 30 seconds). As this apparatus operated when the plant was well illuminated and therefore with the intercellular spaces full of oxygen, the effect of the relatively sudden drops in pressure outside the plant would be similar to injecting bursts of greater pressure into the intercellular spaces. This treatment of speeding up the rhythm in pressure, it was hoped, would show whether pressure was a major factor in determining the form of the plant and especially of the air spaces.

Figs. 7 and 8 show the two adjacent sections of an apical cone of one of these experimental shoots. Very large spaces are apparent in these sections in the layer very close to the tip of the apex. There are also numerous small chinks where three cells come together. These small chinks between the cells at the apex, even among the initials of the apex, have enlarged with the rapidly altering pressure and are more readily visible. Thin chinks forming vertical openings along the tangential cell walls occur frequently, occasionally with short branches in the horizontal plane. After sprigs had been grown for only 1 day under these conditions of alternating pressure, whole fresh apices were examined and even without sectioning these chinks and larger spaces were apparent. The chinks were very much larger than those that normally occur in the rib meristem region under strong light. Both these chinks and the larger holes (about half the size of a cell), appeared dark and were obviously full of gas, not liquid. The extension, enlargement, and fusion of these chinks into larger spaces result from the increased fluctuation in pressure. The outer layers of cells must be under considerable tension, as the gas pressure outside the plant drops quickly, and only a tough layer could avoid rupturing.

In Fig. 8 a prominent metaphase division occurs in the epidermis between the lacuna and the nutrient solution. The dividing cell at certain times would be compressed between the pressure within the lacuna and the pressure in the solution. At other times there must be considerable tension in the cell, tending to extend it vertically when the internal pressure of the plant, especially in the lacunae, is momentarily greater than the external pressure. Thirdly, the cells surrounding a lacuna are favorably placed for gaseous exchange. Undoubtedly a cell situated as the dividing cell should suffer no lack of oxygen during the daylight hours.

The more conventional developments outlined in the first article of this series also occur closer to the tip (as seen by comparing Figs. 7 and 8 of the present article with Fig. 3 of Reference 12). In the internodal tissue, cells round up and split at the corners earlier. The chinks coalesce and enlarge into larger air spaces more rapidly, so that the initiation of the lacunae occurs at about half the usual distance from the tip. In the leaves, the chinks where

three or four cells come together are also greatly enlarged, being three or four times the diameter of those of plants grown under more conventional conditions.

Conclusion

In experimental material grown with and without added oxygen in darkness and light, it has been established that the amount of oxygen relative to the size of the shoot is a major factor influencing the type of air space produced. It has been shown also that changes in gas pressure within the intercellular spaces of the plant do enlarge these spaces so that lacunae can occur very close to the growing tip and in nature such changes are connected with the production of oxygen through photosynthesis, and so with conditions of light and darkness.

Acknowledgment

The author would like to express his thanks to Professor H. B. Sifton for his helpful criticism and suggestions throughout this investigation.

References

1. ALLSOPP, A. Experimental and analytical studies of Pteridophytes. XVII. The effect of various physiologically active substances on the development of *Marsilea* in sterile culture. Ann. Botany, N.S. **16**; No. 62, 165–183 (1952).
2. ALLSOPP, A. Experimental and analytical studies of Pteridophytes. XXIV. Investigations on *Marsilea 4*. Anatomical effects of change in sugar concentration. Ann. Botany, N.S. **18**, No. 72, 449–461 (1954).
3. ALLSOPP, A. Experimental and analytical studies of Pteridophytes. XXVII. Investigations on *Marsilea 5*. Cultural conditions on morphogensis with special reference to the origin of land and water forms. Ann. Botany, N.S. **19**, No. 74, 248–263 (1955).
4. ANGELSTEIN, U. Über die Kohlensäureassimilation submerser Wasserpflanzen in Bikarbonat und Karbonatlösungen. Beitr. Biol. Pflanz. **1**, 86–95 (1911).
5. ARBER, A. Water plants. Cambridge University Press, London. 1920.
6. BECKEL, D. K. B. Cortical disintegration in the roots of *Bouteloua gracilis* (HBK) Lag. New Phytologist, **55**, 183–190 (1956)
7. BLANC, M. LE Sur les diaphragmes des Canaux aérifères des plantes. Rev. gén. botan. T, **24**, 233–243 (1912).
8. BRYANT, A. E. Comparison of anatomical and histological differences between roots of barley grown in aerated and in non-aerated culture solutions. Plant Physiol. **9**, 389–391 (1934).
9. CONSTANTIN, J. Recherches sur la structure de la tige des plantes aquatiques. Ann. sci. nat. (Botan.), Ser. 6, **19**, 285–331 (1884).
10. CORMACK, R. G. H. The development of root hairs by *Elodea canadensis*. New Phytologist, **36**, No. 1, 19–25 (1937).
11. DALE, H. M. Carbon dioxide and root hair development in *Anacharis* (*Elodea*). Science, **114**, 438–439 (1951).
12. DALE, H. M. Developmental studies of *Elodea canadensis* Michx. I. Morphological development at the shoot apex. Can. J. Botany, **35**, 13–24 (1957).
13. DEVAUX, H. Du mecanisme des echanges gazeux chez les plantes aquatiques submergées. Ann. sci. nat. (Botan.), Ser. 7, **9**, 35–179 (1889).
14. DUNN, G. A. Note on the histology of grain roots. Am. J. Botany, **8**, 207–211 (1921).
15. GOEBEL, K. Pflanzenbiologische Schilderungen. Teil 2. Marburg, Leif 2. 1891–93.
16. HOAGLAND, D. R. (1932 and 1933) Mineral nutrition of plants. Ann. Rev. Biochem. **I**, 618–636 (1932); **II**, 471–484 (1933).
17. HULBARY, R. I. The influence of air spaces on the three dimensional shapes in cells in *Elodea* stems and a comparison with pith cells in *Ailanthus*. Am. J. Botany, **31**, 561–580 (1944).

18. KRAMER, P. J. Causes of injury to plants resulting from flooding of soil. Plant Physiol. **26**, 722–735 (1951).

19. MACDOUGAL, D. T. The influence of light and darkness upon growth and development. Mem. N.Y. Botan. Garden, **2**, 1–319 (1903).

20. MCPHERSON, D. C. Cortical air spaces in the roots of *Zea Mays* L. New Phytologist, **38**, No. 3, 190–202 (1939).

21. MYERS, J. The culture of algae for physiological research. *In* The culturing of algae. *Edited by* J. Brunel, G. W. Prescott, and L. H. Tiffany. Kettering Foundation, New York, 1950. pp. 45–51.

22. NORRIS, F. DE LA M. Production of air passages in the root of *Zea Mays* by variation of the culture media. Proc. Bristol Naturalists Soc. Ser. 4, **3**, 134–136 (1913).

23. PRIESTLEY, J. H. and EWING, J. Physiological studies of plant anatomy. VI. Etiolation. New Phytologist, **22**, 30–44 (1923).

24. PRIESTLEY, J. H. The meristematic tissues of the plant. Biol. Revs. Cambridge Phil. Soc. **3**, 1–20 (1928).

25. PRIESTLEY, J. H. Cell growth and cell division in the shoot of the flowering plant. New Phytologist, **28**, 51–81 (1929).

26. SCHENK, H. Vergleichende Anatomie de Submersen Gewächse. Bibliotheca botan. Bd. 1, Heft 1. Kassel. 1886.

27. SCHLOSS-WEILL, B. Über den einfluss des Lichtes auf einige Wasserpflanzen. Beihefte Botan. Centr. **35**, 1–59 (1918).

28. SEVERIN, C. F. Origin and structure of secondary roots of *Sagittaria*. Botan. Gaz. **93**, 93–99 (1932).

29. SIFTON, H. B. Lysigenous air spaces in the leaf of Labrador tea, *Ledum greenlandicum* Oeder. New Phytologist, **39**, 75–79 (1940).

30. SNOW, L. M. Diaphragms of water plants II. Effects of certain factors upon the development of air chambers and diaphragms. Botan. Gaz. **69**, 297–317 (1920).

31. STANT, M. Y. The shoot apex of some monocotyledons. II. Growth organization. Ann. Botany, N.S. **18**, No. 72, 441–447 (1954).

32. STOVER, E. L. The roots of wild rice *Zizania aquatica* L. Ohio J. Sci. **28**, 43–49 (1928).

33. THOMPSON, D'A. W. On growth and form. New ed. Cambridge University Press, London. 1942.

34. TIEGHEM, P. VAN Recherches sur la respiration des plantes submergées. Bull. soc. botan. France, **13**, 411–421 (1866).

35. WATSON, R. W. The effect of cuticular hardening on the form of epidermal cells. New Phytologist, **41**, 223–229 (1942).

36. WIELER, A. Die Function der Pneumathoden und des Aerenchyms. Jahrb. wiss. Botan. **32**, 503–524 (1898).

A COMPARATIVE STUDY OF FUNGITOXICITY AND PHYTOTOXICITY IN AN HOMOLOGOUS SERIES OF N-*n*-ALKYLETHYLENETHIOUREAS[1]

R. G. Ross and R. A. Ludwig

Abstract

A comparative study of fungitoxicity and phytotoxicity was made with an homologous series of N-*n*-alkylethylenethioureas ranging from ethyl to dodecyl. Water solubility decreased and the oil/water partition coefficient increased with increasing length of alkyl side chain. Fungitoxicity increased with increasing chain length up to and including the octyl homologue followed by a sharp decline in the higher members of the series. The more fungitoxic homologues had the same mode of action and the cutoff in toxicity was due to limiting water solubility.

The peak of maximum phytotoxicity depended on the availability of the compounds to the plant cells. As the series was ascended the mobility of the homologues in the plant was found to decrease. When the compounds were readily available to plant cells phytotoxicity increased as the series was ascended up to at least the octyl homologue, but when barriers were present, a peak for phytotoxicity was exhibited near the amyl homologue.

Studies on the uptake of the compounds from aqueous solutions by potato tissue and *Monilinia fructicola* spores showed that a distribution equilibrium existed between the tissue or spores and the external solution. With increasing length of side chain the affinity for the biophase increased and hence the differential toxicity could be attributed to the partitioning of the chemical between the two phases.

The selective action of biocides, as evidenced by their classification as insecticides, fungicides, herbicides, etc., has long been recognized. Differences in selectivity within these broad groups are more subtle but numerous examples of such differences have been observed (1). Many highly active antifungal chemicals fail as agricultural fungicides because they lack the necessary selectivity and cause severe plant injury. The present investigation was undertaken in an attempt to elucidate further some of the properties involved in host–parasite selectivity. An homologous series of N-*n*-alkylethylenethioureas was selected for use in this connection because of anticipated gradients in physical and biological properties, chemical stability, and possibilities of analysis by ultraviolet spectrophotometry.

Literature Review

Interest in ethylenethiourea as a fungicide appears to have begun with its identification as a breakdown product of the ethylenebisdithiocarbamates (4, 12). Barratt and Horsfall (4) examined the toxicity of ethylenethiourea to spores of *Stemphylium sarcinaeforme* and found it to be toxic only at very high concentrations. Rich and Horsfall (17) measured the fungitoxicity of ethylenethiourea, propylenethiourea, and N,N′-di-*n*-butyl-, N-*n*-octyl-, N-*t*-octyl-, and N-*n*-octadecyl-ethylenethiourea to *Stemphylium sarcinaeforme*

[1]Manuscript received October 15, 1956.
Contribution No. 89, Science Service Laboratory, Canada Department of Agriculture, University Sub Post Office, London, Ont. Part of a thesis submitted to the University of Western Ontario by the senior author in partial fulfillment of the requirements for a Ph.D. degree.

and *Monilinia fructicola* using the depression slide technique. N-*n*-octylethylenethiourea was the most toxic of the compounds tested while N-*n*-octadecylethylenethiourea showed some toxicity to both fungi. The other compounds were relatively non-toxic. The fungitoxic derivatives were distinctly more toxic to *M. fructicola* than to *S. sarcinaeforme*. They also found that N-*n*-octylethylenethiourea at 1/400 and 1/1600 severely injured the foliage of young bean and tomato plants.

A number of comprehensive reviews (1, 6, 8, 11, 13, 18) on the variation of biological activity within homologous series and the effects of physical properties on the mode of action of toxicants have already been published, and hence no detailed treatment here is necessary. Sexton (18) lists 11 homologous series in which toxicity has been studied. The number of carbon atoms at which maximum activity occurred varied from 1 to 14. Some were tested against more than one organism and the peaks varied for the different organisms. In a series of alkylthiocyanates maximum toxicity to aphids, for example, was at 10–14 carbon atoms, whereas maximum toxicity to animals occurred at the methyl homologue. He states that "The usual effect in an homologous series is a gradual rise to maximum activity at a certain chain length, followed by a fall as the series is ascended further. This peak effect is often due to the fact that at a certain point, solubility in water becomes a limiting factor". Similarly, Wellman and McCallam (22), studying the effect of substitution of an alkyl group in the 2-position of the glyoxalidine nucleus, found that fungitoxicity increased with chain length until a maximum was reached in the vicinity of 17 carbon atoms, beyond which it fell off. Phytotoxicity increased up to 11 carbon atoms and then dropped off rapidly as the chain length extended beyond 16 carbon atoms. Martin (13) presents data to show that the increase in toxicity with rise in molecular weight, in an homologous series, is usually in a definite geometric progression in which each member is usually about three times as active as its lower homologue.

In relating physical properties of substances to toxicity and the penetration of cytoplasmic membranes, no property has received more attention than the relative solubility of a substance in oil and water. This relative solubility is the foundation for the Lipoid Theory first proposed independently by Overton and by Meyer, who believed that the action of narcotics depended on the suitable partition coefficient between water and lipoid material (1). According to Danielli (9): "Overton found that substances penetrate cells in the same relative order as the order of the oil/water partition coefficients. Since this work of Overton it has been generally assumed that such a correlation indicates the presence of a large proportion of lipoid material in the plasma membrane and that substances with high oil/water partition coefficients penetrate rapidly because they are more soluble in the lipoid layer." The correlation between lipoid solubility, as indicated by the oil/water partition coefficient, and toxicity have been attempted primarily to relate the latter to rate of penetration into cells. Numerous examples

of such a correlation are cited by Horsfall (11). Rich and Horsfall (15, 16) advocate the use of the dosage–response curve to differentiate changes in toxicity due to altered penetration from those due to essential changes in the toxicant. They postulate further that: "Cases where one derivative is most phytotoxic and another derivative most fungitoxic may arise from decided differences in the nature of the lipids of host and parasite. Differences in lipids may also cause differences in the susceptibility of various fungi to the same toxicant."

Physical Properties of Ethylenethiourea and Its Derivatives

Ethylenethiourea and its N-*n*-ethyl, butyl, amyl, hexyl, heptyl, octyl, nonyl, decyl, and dodecyl homologues were used in this work. Their synthesis has already been described (20). Their ultraviolet absorption spectra were determined in glass-distilled water and in absolute ethyl alcohol as a basis for quantitative and qualitative analyses. Aqueous solutions of all the derivatives, with the exception of the N-*n*-decyl and N-*n*-dodecyl, had absorption spectra similar to that of ethylenethiourea itself with a characteristic peak at 232.5 mμ. The curves produced with aqueous solutions of N-*n*-decyl- and N-*n*-dodecyl-ethylenethiourea were erratic, probably because of their low water solubility, and usually contained no characteristic peak. The N-*n*-alkyl derivatives gave characteristic absorption peaks at 242.5 mμ in ethyl alcohol while the peak for ethylenethiourea itself was at 240 mμ.

Water solubilities were determined by shaking an excess of the chemical in glass-distilled water at room temperature until optical densities at 232.5 mμ were constant.

Oil/water partition coefficients were determined by shaking water solutions of known concentrations with an equal volume of oil and subsequently determining the amount of chemical remaining in the aqueous phase. The amount in the oil phase was determined by difference. Olive oil was used in

TABLE I

PHYSICAL PROPERTIES OF ETHYLENETHIOUREA COMPOUNDS

Compound	$E^{1}\%$		Solubility in water, p.p.m.	Oil/water partition coefficient
	Water	Ethyl alcohol		
Ethylenethiourea	1524.2	1561.2	20043	0.016
N-Ethylethylenethiourea	1285.0	1376.2	41011	0.006
N-*n*-Butylethylenethiourea	1076.0	1121.2	3554	0.095
N-*n*-Amylethylenethiourea	983.8	972.6	1832	0.265
N-*n*-Hexylethylenethiourea	917.0	940.0	582	0.850
N-*n*-Heptylethylenethiourea	830.8	841.2	168	3.340
N-*n*-Octylethylenethiourea	768.2	787.5	59	11.910
N-*n*-Nonylethylenethiourea	688.7	741.2	16	42.180*
N-*n*-Decylethylenethiourea		710.0	5*	
N-*n*-Dodecylethylenethiourea		616.7	0.5*	

*Obtained by extrapolation in Fig. 12.

preliminary experiments but resulted in distorted absorption spectra. To avoid this difficulty mineral oil (S.G. = 0.88 at 70° F.) was selected for the oil phase. The absorption spectra of the N-*n*-nonyl determinations gave no indication of the presence of this chemical in the water phase. This does not necessarily mean that all the chemical passed to the oil phase. In subsequent experiments, where aqueous solutions of this compound were shaken in a flask for 2 hours, a decrease in concentration was recorded indicating an adsorption on the glass. The $E^{1\%}$, water solubility, and partition coefficient values are given in Table I.

Fungitoxicity of Ethylenethiourea and Its Derivatives

Fungicidal activity was determined by the slide-germination method essentially as described by the American Phytopathological Society, Committee on Standardization of Fungicidal Tests (2, 3), using *Monilinia fructicola* and *Alternaria solani* as test organisms. The results are presented in Fig. 1 and Table II. Curves were not obtained for the decyl and dodecyl derivatives since, at the necessary concentrations, the large amount of undissolved chemical in the germination drops made it impossible to get accurate germination counts. Toxicity, however, decreased from the nonyl to the dodecyl derivative. The ethyl derivative failed to give complete kill at a dosage of 2000 p.p.m. Ethylenethiourea behaved very erratically. In some trials complete inhibition of germination occurred at 400 p.p.m. while in others some germination occurred at a dosage of 2000 p.p.m. In all assays it was more toxic to *M. fructicola* than N-ethylethylenethiourea.

The dosage–response curves were evaluated by the method of Wilcoxon and Litchfield (23). The analyses showed that all five derivatives differ significantly in potency (19/20 probability) to *M. fructicola* and that the curves are essentially parallel. The results with *A. solani* are very similar except that this fungus is less susceptible to the toxic action of the various derivatives. The dosage–response curves from butyl to octyl are all parallel,

TABLE II

ED_{50} AND SLOPE DATA FROM DOSAGE–RESPONSE CURVES OF N-*n*-ALKYL DERIVATIVES OF ETHYLENETHIOUREA ON THE INHIBITION OF SPORE GERMINATION

Compound	*M. fructicola*		*A. solani*	
	ED_{50} in p.p.m.	Slope*	ED_{50} in p.p.m.	Slope
N-*n*-Butylethylenethiourea	805	1.23	1350	1.07
N-*n*-Amylethylenethiourea	300	1.27	490	1.13
N-*n*-Hexylethylenethiourea	138	1.29	225	1.22
N-*n*-Heptylethylenethiourea	56	1.47	157	1.13
N-*n*-Octylethylenethiourea	38	1.40	80	1.13
N-*n*-Nonylethylenethiourea			235	4.64

*Slope values are in terms of $\frac{ED_{84}/ED_{50} + ED_{50}/ED_{16}}{2}$.

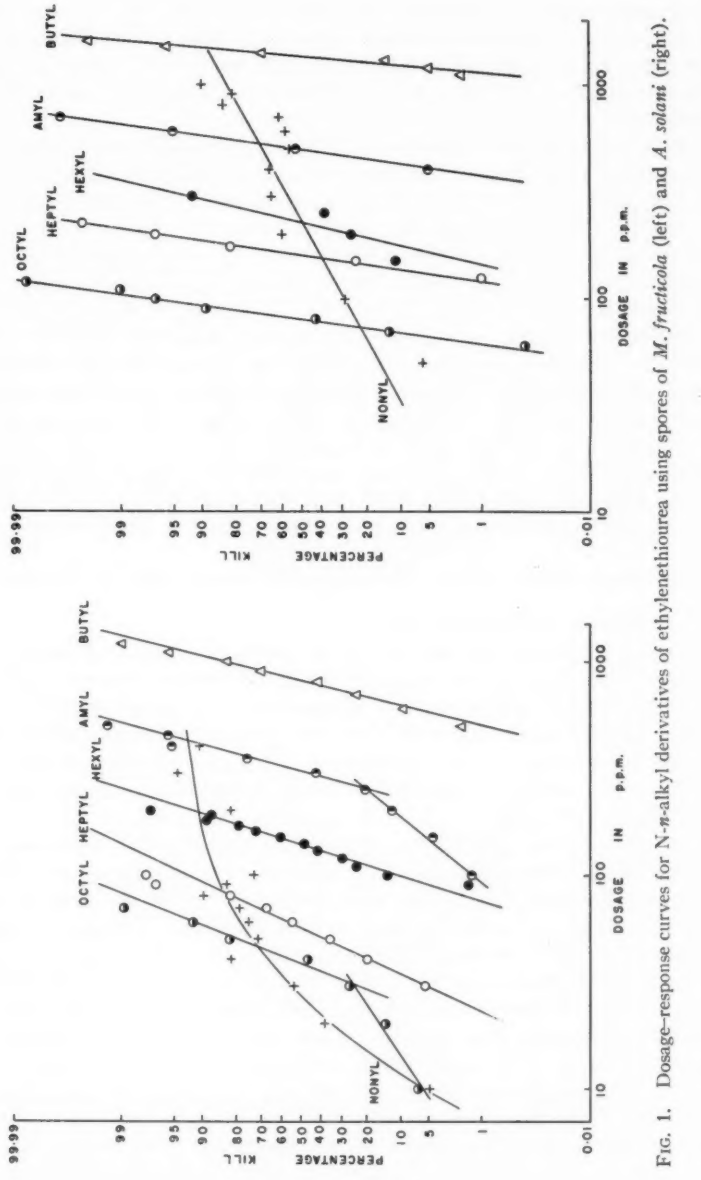


FIG. 1. Dosage–response curves for N-*n*-alkyl derivatives of ethylenethiourea using spores of *M. fructicola* (left) and *A. solani* (right).

and as is readily apparent from Fig. 1, the nonyl curve deviates significantly from parallelism with the other members of the series. Except for N-*n*-hexyl- and N-*n*-heptyl ethylenethiourea, the homologues differ significantly in potency to *A. solani*. The hexyl data are significantly heterogenous, i.e., the line is not a good fit, so large differences in ED_{50} values are needed to be significant.

Rich and Horsfall (17) reported that N-*n*-octadecylethylenethiourea showed some toxicity to *Stemphylium sarcinaeforme* and *Monilinia fructicola*. This finding is at divergence with those reported here. To check these results, a sample of this compound was assayed against *M. fructicola* by the slide-germination method. It showed no toxicity up to 1000 p.p.m. This compound was very difficult to purify because of its wax-like nature, hence Rich and Horsfall's results may have been due to an inadequate purification.

Fungicidal activity was also determined by growing fungi on Czapek's agar to which various concentrations of the chemicals were added. The fungi selected were *M. fructicola*, *Rhizopus nigricans*, *Penicillium expansum*, and *Aspergillus niger*. The results showed that in the homologous series of ethylenethioureas the peak of fungicidal activity remained constant for all four species of fungi. The fungi differed to some extent in their tolerance to the series of chemicals but there was no indication of a shift in the peak of toxicity from the octyl to some other derivative.

Apparent Phytotoxicity of Ethylenethiourea and Its Derivatives

(*a*) *Toxicity to Germinating Seeds*

Studies were made on the toxicity of ethylenethiourea and its derivatives to the germination of two types of seeds: (1) wheat (Cornell) and barley (OAC21), which contain a high concentration of carbohydrates, and (2) radish (scarlet turnip, white top) and flax (variety unknown), which contain a high concentration of oil. If the oil/water partition coefficient plays a role in toxicity it might be expected that the compounds would act differently on the two seed types.

The compounds tested were prepared in solutions or suspensions using a micro wet grinder and diluted to the desired concentrations in 5 ml. quantities of water contained in $\frac{3}{4}$ in. test tubes. Twenty-five of the seeds under test were placed in each tube and held in the refrigerator overnight at approximately 3° C. The seeds and chemical were then transferred to a Petri dish germinator consisting of a 9 cm. Petri dish with a filter paper in the bottom and a side wall liner of cellucotton. The latter functioned as a wick and served to maintain even moisture in the paper without any excess of free liquid. Counts of the seeds failing to germinate were made after a period of 5 days at room temperature. A seed was considered to have germinated if the length of the radicle exceeded half the width of the seed. Untreated seeds almost invariably showed 100% germination so no correction for natural mortality was necessary. The dosage–response curves for N-ethyl-, N-*n*-butyl-, N-*n*-amyl-, and N-*n*-hexyl-ethylenethiourea are given in Fig. 2. Except

for N-*n*-heptylethylenethiourea, the remaining derivatives were non-toxic to all seeds at concentrations up to 4000 p.p.m. With the heptyl homologue, about 4% inhibition of seed germination occurred at dosages ranging from 800 to 4800 p.p.m. The dosage–response curves in Fig. 2 were evaluated by the method of Wilcoxon and Litchfield (23). The ED_{50} and slope data are presented in Table III. For flax and radish the differences in potency for the amyl and hexyl derivatives are not significant (19/20 probability). With barley and wheat the dosage–response curves for the two derivatives deviate significantly from parallelism so a comparison of their ED_{50} values

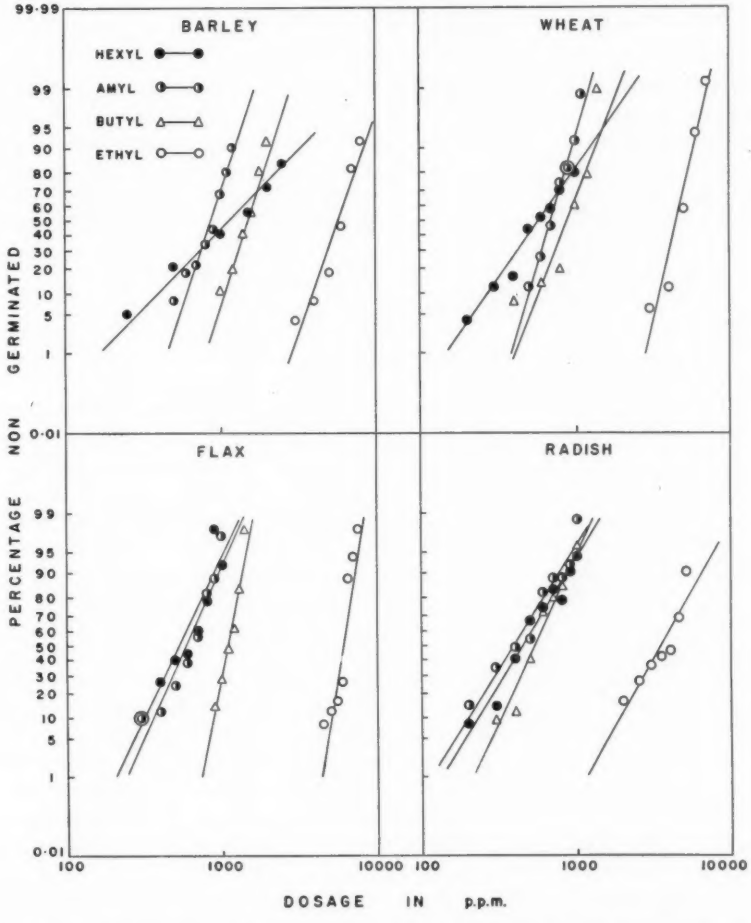


FIG. 2. Dosage–response curves for N-*n*-alkyl derivatives of ethylenethiourea on seeds of various plants.

TABLE III

ED_{50} AND SLOPE DATA FROM DOSAGE–RESPONSE CURVES OF N-*n*-ALKYL DERIVATIVES OF ETHYLENETHIOUREA ON THE INHIBITION OF SEED GERMINATION

Compound	Barley		Wheat		Flax		Radish	
	ED_{50}*	Slope†	ED_{50}	Slope	ED_{50}	Slope	ED_{50}	Slope
N-Ethylethylenethiourea	5700	1.33	4500	1.29	6000	1.15	3500	1.54
N-*n*-Butylethylenethiourea	1500	1.30	890	1.41	1100	1.17	540	1.48
N-*n*-Amylethylenethiourea	870	1.33	700	1.29	590	1.45	395	1.72
N-*n*-Hexylethylenethiourea	1140	2.28	590	1.81	540	1.51	450	1.69

*p.p.m.

†Slope values are in terms of $\frac{ED_{84}/ED_{50} + ED_{50}/ED_{16}}{2}$.

is not feasible. The data indicate that the amyl derivative is the more toxic to both species of seed. There is little indication that differences in oil or lipoid content of the different seed types affects their relative behavior to the series since differences in tolerance to the compounds do not appear to be correlated with seed type. The more abrupt change in slope that occurs between the amyl and hexyl homologues with the carbohydrate seeds may be of some significance.

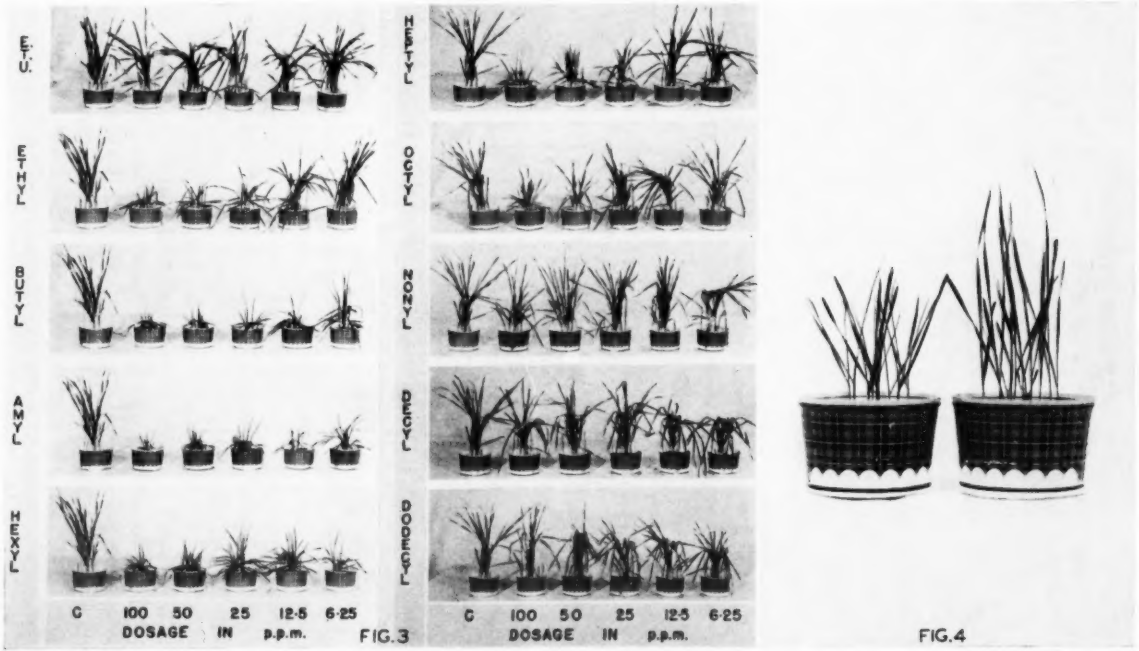
(*b*) *Toxicity to Growing Plants*

The results reported in the previous section show that, in the homologous series of ethylenethioureas, toxicity to seed germination increases as the series is ascended from the ethyl to the amyl or hexyl homologue beyond which there is an abrupt decrease in toxicity. The following experiments were carried out to determine if this peak of toxicity is also exhibited with established plants of tomato (John Baer), wheat, flax, and radish.

The plants were grown in sand in 1 pint waxed paper containers and treatments begun when the plants were well established. The compounds in solution or suspension were applied to the sand in a geometric dilution series, 50 ml. of each dilution being added daily to two containers of each species for 10 days. Check plants received an equivalent amount of water. Precautions were taken when chemicals were added to ensure that they did not come in contact with the foliage of the plants.

The photographs of wheat in Fig. 2 show that N-*n*-amylethylenethiourea was the most phytotoxic member of the series. Similar results were obtained with the other plant species. Ethylenethiourea itself was non-toxic. The homologues were increasingly more toxic as the series was ascended from ethyl to amyl. At the hexyl derivative toxicity began to decrease until at the octyl or nonyl homologue, depending on the tolerance of the plant species, the derivatives produced no apparent signs of phytotoxicity. Tomatoes were the least tolerant to the compounds and even at 2.5 p.p.m. the butyl and amyl derivatives caused a slight stunting. The symptoms of injury to any one plant species were constant throughout the toxic members of the series and varied only in severity. The injury ranged from a slightly stunted condition

PLATE I



FIGS. 3 AND 4. Comparative toxicity of ethylenethiourea (E.T.U.) and its N-*n*-alkyl derivatives to wheat. Fig. 3 shows effect of a dilution series of the various homologues on wheat while Fig. 4 shows the typically curled leaves of treated plants on the left and normal plants on the right.

Ross and Ludwig—Can. J. Botany

through various degrees of chlorosis, tip and marginal necrosis, and distortion of the foliage to a condition of moribundity. A marked change in the type of growth was very apparent in the wheat plants. The margins of the secondary leaves curled upward and became very tightly rolled resembling an onion leaf (see Fig. 4). These symptoms have been described on wheat by Slykhuis (19) in investigations on the relation of a mite of the genus *Aceria* to wheat streak mosiac.

Movement of Ethylenethiourea and Its Derivative Through Intact Root Systems

The permeability of cells to ethylenethiourea and its derivatives was examined by comparing the flow of solutions of the different homologues through uninjured root systems. The endodermis of the root constitutes an unbroken layer of cells through which water and solutes must pass. John Baer tomato plants were grown in nutrient solution until they had developed an extensive root system and attained a first internode diameter of about 8 mm. This took from 1 to 2 months depending on the time of year. The root systems were cut off about 1 in. above the crown with a sharp razor blade, rinsed in distilled water, and placed in the appropriate solutions in 500 ml. beakers. The stumps were attached with rubber tubing to side-arm test tubes via inverted capillary U tubes and the connections made airtight by being bound tightly with strips of 'Dental Dam'. Each of the tubes with its attached root system was clamped in a vertical position and adjusted so that the root was immersed and the union was above the liquid. The side arms of the test tubes were then connected with vacuum tubing to a manifold, and liquid was drawn through the roots by applying a negative pressure varying between 40 and 60 cm. of mercury.

In all experiments the initial rate of flow of water was determined and the roots then transferred to a 10 p.p.m. solution of the appropriate homologue. The initial 10 ml. quantity of the solution passing through the root was discarded and the second 10 ml. aliquot was collected for analysis. Following this the roots were returned to distilled water and the rates of flow were redetermined. The samples collected for analysis were dried under reduced pressure at 40° C. and extracted with three 2-ml. aliquots of ether; the ether was evaporated under a steam of nitrogen, and the ether residue dissolved in ethyl alcohol for ultraviolet analysis. The concentrations of the homologues were calculated from the optical density at their respective peaks minus that of the water control at the same wave length. The method of analysis was not fully quantitative since only 80% recovery was obtained from samples of known concentration. In spite of these inaccuracies the results from any one experiment should be comparative and show any large differences in the movement of the compounds through the root systems.

There was no indication that the compounds had any effect on permeability as measured by the rate of movement of liquid through the root systems. Passage through the root systems decreased the concentrations of all the

derivatives but in all determinations, this concentration decrease was greater for the octyl derivative than for the lower members of the series. This is shown in the averaged data presented in Table IV.

In view of these results a series of experiments were undertaken in which the removal of the chemicals from solution by tomato plants was studied.

Tomato plants were grown in nutrient solution for this purpose. When they had reached a height of about 2 ft., their roots were rinsed in distilled water and placed in 250-ml. Berzelius beakers containing 200 ml. of 10 p.p.m. solutions of N-ethyl-, N-*n*-amyl-, or N-*n*-octyl-ethylenethiourea. Control solutions consisted of 200 ml. of water. Each treatment was replicated four times. The plants were grown in these solutions for 4 days. On each

TABLE IV

AVERAGE CONCENTRATIONS OF ETHYLENETHIOUREA COMPOUNDS IN SOLUTIONS PASSING THROUGH TOMATO ROOT SYSTEMS

Compound	No. of determinations	Concentration, p.p.m.
Ethylenethiourea	2	4.0
N-Ethylethylenethiourea	7	3.8
N-*n*-Butylethylenethiourea	3	5.8
N-*n*-Amylethylenethiourea	6	4.7
N-*n*-Hexylethylenethiourea	3	5.3
N-*n*-Heptylethylenethiourea	1	5.4
N-*n*-Octylethylenethiourea	7	2.4

day the levels of the solutions in the beakers were restored and the uptake of solution recorded. After 4 days the concentration of each solution was determined by ultraviolet spectrophotometry using water from the controls in the reference cell to cancel out any ultraviolet absorption by substances from the root systems. The spectra of the controls remained fairly constant throughout and characteristic spectra were obtained for the ethylenethiourea derivatives.

Accumulation in or on the roots was also determined. The root systems were rinsed in distilled water and the lateral roots cut off and freeze-dried. The dry roots were then ground in a mortar to a fine powder and 0.1 g. of this powder extracted with 10 ml. of absolute ethyl alcohol. This was done by adding the alcohol to the powder, agitating the mixture for about 5 minutes, and removing the powder by centrifugation. The supernatant was then analyzed spectrophotometrically using the control extract in the reference cell. These quantities should be considered relative rather than absolute owing to the impure state of the extracts.

The results of this experiment, given in Table V, are presented as the average of the four replicates. Despite the crude analytical method the results show definitely that the roots contained considerable quantities of N-*n*-octylethylenethiourea, a lesser quantity of N-*n*-amylethylenethiourea, and no detectable quantity of N-ethylethylenethiourea. Since the three

TABLE V

ABSORPTION OF N-ETHYL-, N-*n*-AMYL-, AND N-*n*-OCTYL-ETHYLENETHIOUREA BY TOMATO PLANTS IN WATER CULTURE

Compound	Water added to maintain constant volume, ml.	Uptake from solution in γ	γ recovered from 0.1 g. of roots
N-Ethylethylenethiourea	147	1040	0
N-*n*-Amylethylenethiourea	149	1180	9.6
N-*n*-Octylethylenethiourea	127	1054	95.7
Water	160		

ethylenethiourea derivatives disappeared equally from the external solution, it must be concluded that differential transport occurs in the plant. Apparently N-*n*-octylethylenethiourea readily accumulates in or on the root system, the amyl derivative accumulates to a much lesser extent, and the ethyl derivative is easily transported out of the root system. Although the results indicate some differences in the volume of solution absorbed, this appeared to be more a function of the individual plants than any effect of the solutes on solution uptake.

It is evident, therefore, that the decapitated root technique used in the preceding experiment measured differences in translocation of the ethylenethioureas through tomato roots and not necessarily the permeability of plant cells to the various homologues.

Uptake of Ethylenethiourea and Its Derivatives from Aqueous Solutions by Plant and Fungal Cells

It has been shown that the ethylenethiourea homologues are differentially accumulated in the roots of tomato plants. This suggests that the absorptive, or adsorptive, forces of the members of the homologous series may affect their translocation in plants or tissue. It becomes apparent that one of these sorptive forces may be important in determining the factor, or factors, responsible for phytotoxicity and fungitoxicity in this homologous series of ethylenethioureas. Consequently, studies were carried out on the uptake of the homologues from aqueous solutions by plant tissue and fungal cells.

Uptake of Ethylenethiourea and Its Derivatives from Aqueous Solutions by Potato Tissue

In these experiments, 4 mm. cubes of potato tuber tissue were washed in running tap water for at least 3 hours and the excess water was removed from the surface by blotting the cubes with paper towels. Five grams of cubes was added to each of two 200-ml. Erlenmeyer flasks containing 15 ml. of a 20 p.p.m. ($\pm$ 0.5) solution of the appropriate ethylenethiourea. A third check flask contained chemical without potato. Controls consisting of potatoes in distilled water were also included. All flasks received gentle shaking at 23° C. The solutions were then analyzed spectrophotometrically.

In determining the concentrations of the solutions which had contained potato, the water control was used in the reference cell of the spectrophotometer in order to compensate for substances diffusing from the potato tissue. The ultraviolet absorption spectra of these control solutions were always almost identical and remained constant from one experiment to the next. The assumption was made that the substances diffusing from the potato were the same in water and in ethylenethiourea solutions. Since characteristic spectra were obtained of all the compounds, this assumption seemed to be justified. The uptake of the compounds by the potato tissue was calculated from the difference in concentration between solutions containing potato and the check solutions.

Preliminary experiments indicated a gradient in the homologous series of ethylenethioureas with regard to the quantity taken out of solution by potato tissue. An experiment with the ethyl, amyl, and octyl derivatives in which solutions were analyzed at intervals of 2 and 3 hours showed that there was no appreciable further uptake after 2 hours. A second experiment was carried out with N-*n*-octylethylenethiourea in which the solutions contained potato tissue for 0.25, 0.5, 1, 2, and 3 hour intervals. The results are presented in Fig. 5. They show that the rate of uptake is rapid in the first 15 minutes after which it declines until between 2 and 3 hours it becomes relatively insignificant. In view of these results an uptake period of 2 hours was adopted for subsequent tests.

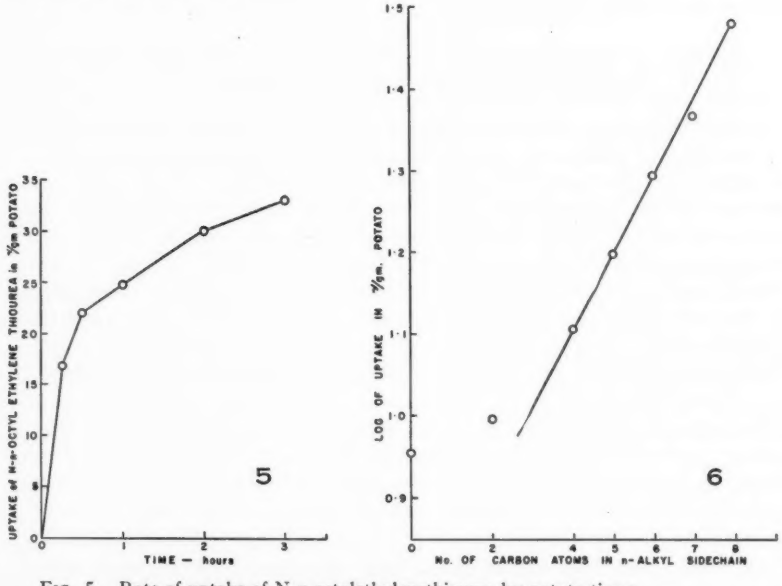

UPTAKE of N-n-OCTYL ETHYLENE THIOUREA in γ/gm. POTATO
TIME — hours
5
LOG OF UPTAKE IN γ/gm. POTATO
No. OF CARBON ATOMS IN n-ALKYL SIDECHAIN
6

FIG. 5. Rate of uptake of N-*n*-octylethylenethiourea by potato tissue.
FIG. 6. Uptake of ethylenethiourea (○) and its N-*n*-alkyl derivatives by potato tissue from 20 p.p.m. aqueous solutions. (Line calculated on a geometric progression of 1.24.)

The uptake of various members of the series by potato tissue was next examined. In this experiment an attempt was made to determine also the amount of chemical present in the tissue. This was done to find if the compounds were reacting with substances in the potato tissue or were taken up unchanged. In making these determinations the potato cubes from the two flasks containing each chemical were bulked, freeze-dried, and ground in a mortar to a fine powder. This powder was taken up in 10 ml. of absolute ethyl alcohol, agitated for 5 minutes, and removed by centrifugation. The supernatant was then analyzed spectrophotometrically using the extract of the potato in the water control in the reference cell. As with previous analyses of this type, the results are not fully quantitative.

TABLE VI

COMPARATIVE UPTAKE OF ETHYLENETHIOUREA AND ITS DERIVATIVES BY POTATO TISSUE

Compound	Uptake in γ/g. of potato	Recovery from potato in γ/g. of potato
Ethylenethiourea	9.00	
N-Ethylethylenethiourea	9.90	0.18
N-*n*-Butylethylenethiourea	12.80	1.13
N-*n*-Amylethylenethiourea	15.80	2.26
N-*n*-Hexylethylenethiourea	19.70	2.42
N-*n*-Heptylethylenethiourea	23.30	8.79
N-*n*-Octylethylenethiourea	30.20	12.28

The results of this experiment are presented in Fig. 6 and Table VI. The figures given on uptake are the average of two replicates carried out on different days. Those on recovery are from the first replicates. The concentration of saturated solutions of the nonyl derivative without potato decreased so it was excluded from the tests.

The results show that as the homologous series is ascended the uptake by potato tissue increases. The compounds were recovered from the potato tissue in the same order as they were taken out of solution. Only a small part of that taken out of solution was recovered from the potato tissue. The proportion is greater with the higher members of the series, which might indicate that a larger quantity of the more water-soluble members of the series was lost by rinsing of the cubes in distilled water. Fig. 6 shows that from butyl to octyl the series exhibits a regular geometric increase, the quantity of each member taken up being 1.24 times that of its lower homologue.

Factors Affecting the Uptake of N-n-Amyl- and N-n-Octyl-ethylenethiourea by Potato Tissue

To ascertain if the uptake of the ethylenethiourea compounds by potato tissue is dependent on the permeability of the protoplasts to the various compounds, studies were carried out on the effect of various factors on uptake by potato tissue. N-*n*-amyl- and N-*n*-octyl-ethylenethiourea were selected as representative members of the series.

(*a*) *Salts*

The effect of salts on the uptake by potato tissue was determined by mixing equal volumes of a 40 p.p.m. solution of the ethylenethiourea compound and double strength solutions of 0.1 and 0.01 *M* chloride salts of the mono-, di-, and tri-valent cations, Na^{+}, Ca^{++}, and Al^{+++}. Control solutions consisting of duplicate lots of potato tissue in 0.1 and 0.01 *M* solutions of each salt were included. The absorption spectra of these duplicate control solutions were always almost identical. The salts themselves had no effect on the normal spectra of the ethylenethiourea compounds as evidenced by analyses on 20 p.p.m. solutions of the homologues with and without potato and salt solutions of the homologues without potato.

The results presented in Table VII are the average of two replicates carried out on the uptake of the ethylenethiourea compounds by potato tissue.

TABLE VII

EFFECT OF SALTS ON THE UPTAKE OF N-*n*-AMYL- AND N-*n*-OCTYL-ETHYLENETHIOUREA BY POTATO TISSUE

Salt	Molarity	Amyl	Octyl
Sodium chloride	0.1	15.2*	29.5
	0.01	16.5	29.6
Calcium chloride	0.1	15.0	30.3
	0.01	13.9	29.7
Aluminum chloride	0.1	13.9	27.8
	0.01	10.7	28.4
Check		14.6	29.6

*Uptake in γ/g. of potato tissue.

(*b*) *pH*

The procedure for determining the effect of pH on the uptake of the ethylenethiourea compounds was the same as that in the preceding experiment, except that a series of buffer mixtures was substituted for the salts. Buffers were selected that had no effect on the normal absorption spectra of the homologues. In one experiment buffer mixtures of sodium monohydrogen phosphate and potassium dihydrogen phosphate, resulting in pH values of 5.4, 6.6, and 7.7, were used (7). In a second experiment, buffer mixtures, with pH values of 5.0, 6.6, and 8.4, were obtained by titrating together 0.1 *M* solutions of sodium pyrophosphate and hydrochloric acid. The results are given in Table VIII.

The results in Table VIII indicate that, in the physiological range, uptake of the ethylenethiourea homologues is not affected by pH. Experiment 2 shows a slight increase in uptake with increase in pH but this does not occur in Experiment 1, so it is probably due to other factors.

(*c*) *Temperature*

Measurements on uptake by potato tissue were made at three different temperatures, viz. 10°, 22°, and 32° C. The solutions were at the various temperatures before the addition of the potato cubes. They did not receive

TABLE VIII

EFFECT OF pH ON THE UPTAKE OF N-*n*-AMYL- AND N-*n*-OCTYL-ETHYLENETHIOUREA BY POTATO TISSUE

Experiment	pH	Amyl	Octyl
1	5.4	17.5*	31.3
	6.6	15.0	28.6
	7.7	15.2	30.9
	Check	15.8	29.7
2	5.0	17.6	33.3
	6.6	18.7	36.0
	8.4	20.4	39.7
	Check	17.1	31.9

*Uptake in γ/g. of potato tissue.

continuous agitation but were shaken for 1 minute at 30 minute intervals. The experiment was replicated on two different days, and the results in Table IX are the average of the two replicates. In this range, temperature has no measurable effect on the uptake of the ethylenethiourea homologues by potato tissue.

TABLE IX

EFFECT OF TEMPERATURE ON THE UPTAKE OF N-*n*-AMYL- AND N-*n*-OCTYL-ETHYLENETHIOUREA BY POTATO TISSUE

Temperature	Amyl	Octyl
10° C.	10.8*	20.7
22° C.	12.3	21.4
32° C.	12.7	21.8

*Uptake in γ/g. of potato tissue.

(*d*) *Oxygen*

A comparison was made of the uptake of N-*n*-amyl- and N-*n*-octyl-ethylenethiourea in atmospheres of nitrogen and air. Potato cubes were prepared in the usual manner and those to be tested under nitrogen were preconditioned by a 30 minute period in water through which nitrogen was bubbled vigorously. Air was also removed from solutions to be tested under nitrogen prior to the addition of tissue. The average results of the experiments duplicated on different days are given in Table X. Apparently oxygen is unnecessary for the uptake of the ethylenethiourea homologues by potato tissue.

TABLE X

EFFECT OF OXYGEN ON THE UPTAKE OF N-*n*-AMYL- AND N-*n*-OCTYL-ETHYLENETHIOUREA BY POTATO TISSUE

	Amyl	Octyl
Oxygen	13.6*	31.7
Nitrogen	14.3	33.4

*Uptake in γ/g. of potato tissue.

(*e*) *Solution Concentration*

The effect of solution concentration on the uptake of N-*n*-amyl- and N-*n*-octyl-ethylenethiourea was measured by adding 5 g. of potato cubes to 15 ml. of 50, 40, 30, 20, and 10 p.p.m. solutions of the two compounds. The procedure followed was that already outlined. The results as presented in Table XI shows that the proportion of uptake is independent of solution concentration. This implies that an equilibrium or partition exists between solutions of the compound and the potato tissue.

TABLE XI

EFFECT OF SOLUTION CONCENTRATION ON THE UPTAKE OF N-*n*-AMYL- AND N-*n*-OCTYL-ETHYLENETHIOUREA BY POTATO TISSUE

	A_1/A_2*	
Concentration, p.p.m.	Amyl	Octyl
50	0.31	1.00
40	0.31	1.07
30	0.37	1.03
20	0.32	1.05
10	0.31	1.04

*A_1—compound taken up by potato tissue.
A_2—compound remaining in solution.

Partitioning of N-n-Octylethylenethiourea Between Solution and Potato Tissue

An experiment was undertaken to determine if there is a definite equilibrium or distribution between potato tissue and solutions of N-*n*-octylethylenethiourea. Five grams of potato cubes were placed in 15 ml. of a 50 p.p.m. solution of N-*n*-octylethylenethiourea. At 2- and 4-hour intervals, 7.5 ml. of solution was withdrawn from the flasks and replaced with 7.5 ml. of distilled water. After 6 hours the potato was removed from the solutions and analyses carried out on all samples. The experiment was done in quadruplicate and included appropriate control solutions.

The results of two experiments are given in Table XII. The expected concentrations are calculated from the determined concentration in control flasks lacking potato using a value for A_1/A_2 of 1 (see Table XI). For example,

TABLE XII

PARTITIONING OF N-*n*-OCTYLETHYLENETHIOUREA BETWEEN SOLUTION AND POTATO TISSUE

Experiment	Dilution	Obtained	Expected*
1	0	25.0	23.4
	1	15.5	17.1
	2	11.3	13.3
2	0	26.8	25.9
	1	15.9	19.2
	2	11.3	15.2

*Concentration of solution in p.p.m.

at 0 dilution, the compound should be equally partitioned between solution and potato. At dilution 1, when the solution is diluted ½, the compound taken up by the potato at 0 dilution and that in solution should again become equally partitioned. The results suggest that this does occur. At dilutions 1 and 2 the obtained values are lower than the expected, which might indicate that a longer period of time is required for the compound to establish an equilibrium when it must move from the potato into solution. Thus the differential uptake from solution of the ethylenethiourea homologues by potato tissue appears to be a distribution phenomenon dependent on the partition coefficient of the homologues between tissue and water.

Uptake of Ethylenethiourea and Its Derivatives from Aqueous Solutions by Cellulose

As an indication of the affinity of the homologous series of ethylenethioureas for cellulose, the chief constituent of cell walls, determinations were made on the uptake or adsorption of the various homologues by filter paper. The technique used was the same as with potato tissue. One gram samples of filter paper cut into 0.25 in. squares were placed in 15 ml. quantities of 20 p.p.m. solutions of the various homologues for a period of 2 hours. They were then removed by centrifugation and the solutions analyzed. The results are given in Fig. 7 and show that the affinity for cellulose increases in

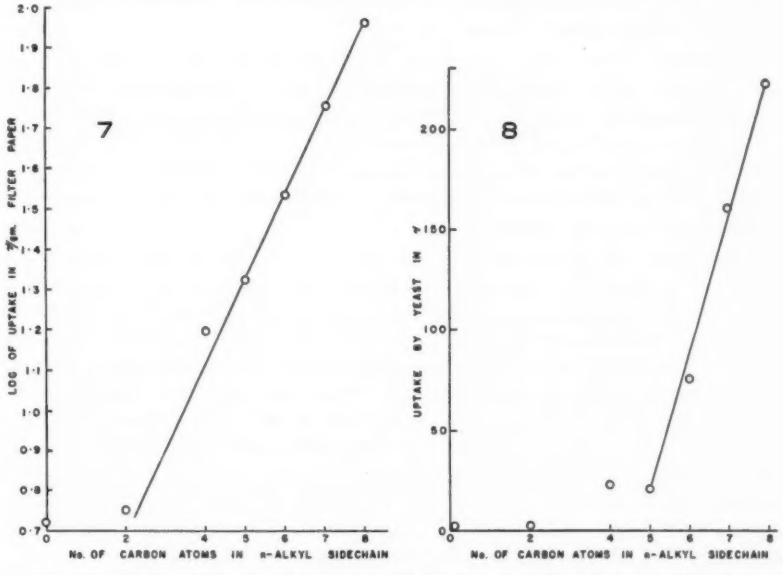

7
LOG OF UPTAKE IN γ/gm. FILTER PAPER
No. OF CARBON ATOMS IN n-ALKYL SIDECHAIN
8
UPTAKE BY YEAST IN γ
No. OF CARBON ATOMS IN n-ALKYL SIDECHAIN

FIG. 7. Uptake of ethylenethiourea (○) and its N-*n*-alkyl derivatives by filter paper from 20 p.p.m. aqueous solution. (Line calculated on a geometric progression of 1.64.)
FIG. 8. Uptake of ethylenethiourea (○) and its N-*n*-alkyl derivatives by yeast from 20 p.p.m. aqueous solutions. (Line calculated on an arithmetic progression of 67.4.)

a geometric ratio as the series is ascended. From the amyl to the octyl homologue the average ratio is 1.64, i.e. the quantity of each member adsorbed is 1.64 times that of its lower homologue.

Uptake of Ethylenethiourea and Its Derivatives by Yeast

A Fleischmann's Yeast cake was suspended in distilled water, and the cells were washed three times by centrifugation and resuspended in 150 ml. of distilled water. Seven and one-half milliliters of this suspension was added to each of two 200-ml. Erlenmeyer flasks each containing 7.5 ml. of a 40 p.p.m. solution of the appropriate ethylenethiourea. A third check flask contained 15 ml. of the compound at 20 p.p.m. Water controls for use in the spectrometer consisted of three flasks containing the same quantity of yeast in 15 ml. of distilled water. The solutions received gentle shaking for 2 hours at 23° C. Following this the yeast cells were removed by centrifugation and the supernatant analyzed as previously described.

Fig. 8 shows the average results of two experiments conducted on different days. Ethylenethiourea and N-ethylethylenethiourea were taken up by yeast, butyl and amyl were taken up in very small quantities, and, as the series ascends from amyl to octyl, uptake increases in an average arithmetic progression of 67.4 for each member of the series.

Uptake of Ethylenethiourea and Its Derivatives from Aqueous Solutions by Monilinia fructicola Spores

It has been shown that a partition coefficient exists between potato tissue and solutions of the ethylenethiourea compounds. This partition coefficient increases as the series ascends. Toxicity to *Monilinia fructicola* also increases as the series ascends up to the octyl homologue, which indicates that a correlation may exist between toxicity and the uptake of the compounds. Therefore studies were carried out on the uptake of various members of the homologous series by spores of *M. fructicola*.

The fungus was grown on V-8 juice agar (14). After an incubation period of about 30 days the spores were removed by flooding the cultures with distilled water, rubbing them lightly with a rubber policeman, straining the suspension through cheesecloth to remove the larger mycelial fragments, washing the spores three times by centrifugation, and resuspending them in the desired quantity of distilled water. Two and one-half milliliters of this spore suspension were added to each of two 30 ml. beakers each containing 2.5 ml. of a 40 p.p.m. solution of the appropriate ethylenethiourea. A third check beaker contained 5 ml. of the compound at 20 p.p.m. Three control beakers contained the same number of spores in 5 ml. of distilled water. After 2 hours' gentle shaking at room temperature the spores were removed by centrifugation, and analysis carried out on the supernatant.

The experiment was duplicated on different days with a final spore concentration of about 10,000,000 per ml. in each instance. The average results of these experiments are given in Table XIII.

TABLE XIII

COMPARATIVE UPTAKE OF ETHYLENETHIOUREA AND ITS DERIVATIVES BY *M. fructicola* SPORES

Compound	Uptake in /1,000,000 spores
Ethylenethiourea	0
N-Ethylethylenethiourea	0
N-*n*-Butylethylenethiourea	0
N-*n*-Amylethylenethiourea	0
N-*n*-Hexylethylenethiourea	0.70
N-*n*-Heptylethylenethiourea	0.93
N-*n*-Octylethylenethiourea	2.61

They indicate a gradient in the series for the uptake of *M. fructicola*.

Effect of Solution Concentration on the Uptake of N-n-Octylethylenethiourea by Spores of M. fructicola

The effect of solution concentration was determined using essentially the same methods as in the preceding experiment. Spores were added to solutions of N-*n*-octylethylenethiourea resulting in solution concentrations of 40, 30, 20, and 10 p.p.m. In the first experiment the final spore concentration was 7,000,000 per ml., and in the second, 11,000,000 per ml. The results of these two experiments, carried out on different days, are given in Table XIV.

TABLE XIV

EFFECT OF SOLUTION CONCENTRATION ON THE UPTAKE OF N-*n*-OCTYLETHYLENETHIOUREA BY *M. fructicola* SPORES

	A_1/A_2*	
Concentration, p.p.m.	Experiment 1	Experiment 2
40	0.30	0.30
30	0.30	0.31
20	0.31	0.45
10	0.35	0.53

*A_1—compound taken up by spores.
A_2—compound remaining in solution.

These results imply that a partitioning also exists between solutions of N-*n*-octylethylenethiourea and fungal spores.

Partitioning of N-n-Octylethylenethiourea between Solution and M. fructicola Spores

The methods used were those of the preceding spore uptake tests combined with the dilution technique of the corresponding experiment with potato tissue. Each experiment was done in quadruplicate, starting with a solution concentration of approximately 40 p.p.m. At 2 and 4 hour intervals, the

spores were centrifuged out, 2.5 ml. of solution withdrawn, 2.5 ml. of distilled water added, and the spores resuspended. The spore concentration in the first and second experiments was 7,000,000 and 11,000,000 per ml., respectively. The results of these two experiments, carried out on different days, are given in Table XV. The expected concentrations are calculated from a value for A_1/A_2 of 0.3 (see Table XIV). The concentrations obtained agree with

TABLE XV

PARTITIONING OF N-*n*-OCTYLETHYLENETHIOUREA BETWEEN SOLUTION AND *M. fructicola* SPORES

Experiment	Dilution	Obtained	Expected*
1	0	30.2	29.0
	1	17.8	18.5
	2	11.0	12.2
2	0	31.8	30.5
	1	18.9	19.4
	2	11.6	12.8

*Concentration of solution in p.p.m.

those expected, establishing a definite partitioning of the compound between solution and spores of *M. fructicola*. As in potato tissue the differential uptake of the ethylenethiourea homologues by spores appears to be a distribution phenomenon dependent on the partition coefficient of the homologues between spores and water.

Actual Phytotoxicity of Ethylenethiourea and Its Derivatives

The results reported thus far have shown a peak in the homologous series for phytotoxicity at or near the amyl homologue and one for fungitoxicity at the octyl homologue. The ethyl, amyl, and octyl derivatives were taken up by tomato plants in about equal quantities but differed in their mobility in the plant. The homologues also differ in their affinity for plant tissue. This suggests that translocation or adsorption in the plant may be a factor in determining the peak of phytotoxicity in this homologous series. An attempt was therefore made to study phytotoxicity, making allowance for translocation or adsorption by plant tissue.

Duplicate experiments were run in which excised tops of 6 to 8 in. high John Baer tomato plants were allowed to take up 30 p.p.m. aqueous solutions of representative homologues through their cut stem ends. The cuttings were held in randomized block in the greenhouse throughout the experiment. Results were recorded by observing the condition of the plants and measuring their height at the end of each experiment. The latter was taken from the cut end of the stem to the top node. In Experiment 1 this was recorded as the total height of each plant at the end of the experiment and in Experiment 2 as the increase in height from the beginning to the end of the experiment. These results are given in Table XVI.

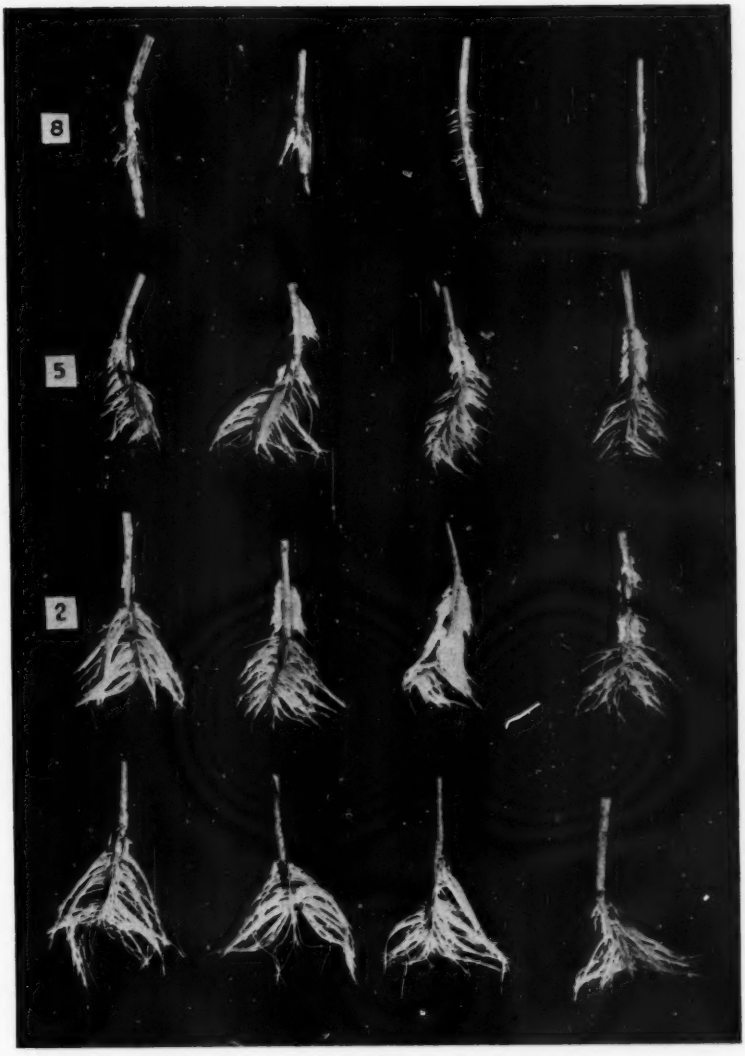


FIG. 9. Adventitious root systems from excised tomato plants grown in 30 p.p.m. solutions of N-*n*-octyl- (8), N-*n*-amyl- (5), and N-ethyl-ethylenethiourea (2). Bottom row—checks.

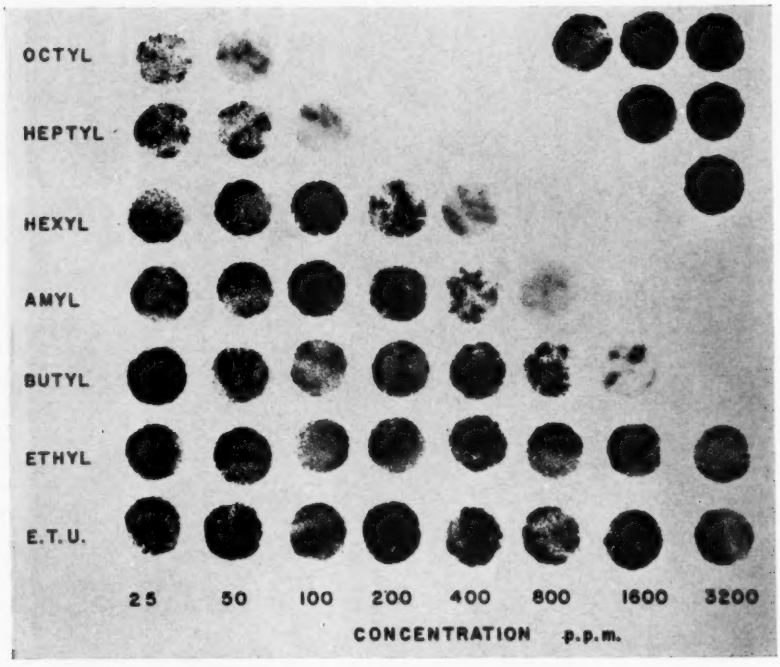


FIG. 10. Comparative toxicity of ethylenethiourea (E.T.U.) and its N-*n*-alkyl derivatives to potato tuber disks. Checks—upper right.

TABLE XVI

EFFECT OF THREE ETHYLENETHIOUREA HOMOLOGUES ON THE GROWTH OF EXCISED TOMATO PLANTS

Compound	Experiment 1*	Experiment 2†
N-Ethylethylenethiourea	20.0	2.6
N-*n*-Amylethylenethiourea	19.1	2.3
N-*n*-Octylethylenethiourea	16.3	1.7
Control	23.0	4.7

*Average height of plants in cm. L.S.D. between treatments: 5% level, 2.5; 1% level, 3.3.
†Average increase in height of plants in cm. L.S.D. between treatments: 5% level 1.5; 1% level, 2.0.

All three compounds significantly depressed the growth of the excised tomato plants. Growth in the octyl solution was significantly less than that in ethyl or amyl. These differences in growth were obviously partly due to the effect of the compounds on the development and growth of adventitious roots. Fig. 9 shows representative root systems from each treatment. N-*n*-Octylethylenethiourea almost completely inhibited root development, N-*n*-amyl to a lesser extent, and only slight inhibition occurred with N-ethylethylenethiourea. It is interesting to note that the ethyl and amyl homologues stimulated root-hair development.

Except for stunting, the most serious injury to the tops of the plants was caused by the ethyl and amyl homologues. This injury was in the form of a curling and distortion of the new growth, a general chlorosis of the older foliage, beginning at the margins and tips and progressing inward, resulting in death and abscission of the more severely injured leaves. The amyl derivative seemed to be slightly more toxic than the ethyl. Very little distortion or chlorosis occurred in the octyl treated plants although there was longitudinal cracking of the stems and petioles which resulted in necrotic streaks. This type of injury was not observed in the other treatments. The chlorotic condition of the foliage demonstrated the difference in the movement of the compounds to the top of the plants. Chlorosis was more advanced in the ethyl treated plants indicating that it was more rapidly translocated to the leaves. There was practically no chlorosis in the octyl treated plants while those from the amyl treatment were only slightly chlorotic at the margins. This difference in the movement or availability of the compounds was also apparent in the time of abscission of the lower petioles. Abscission occurred 2 or 3 days earlier in the ethyl treated plants than in those in solutions of N-*n*-amylethylenethiourea, whereas very little abscission occurred with plants in N-*n*-octylethylenethiourea. The results of these experiments indicate that the octyl rather than the amyl homologue is the more phytotoxic member of the series to those parts of the plant in which it is readily available.

In another series of experiments, tomato plants, grown singly in 4-in. clay pots of soil until about 6 in. high, were sprayed to 'runoff' with suspensions or solutions of the ethylenethiourea compounds, at dilutions of 2000, 1000, 500, and 250 p.p.m. Two definite and distinct types of phytotoxicity were recorded, one for the homologues from ethyl to heptyl and another for those from octyl to dodecyl. Ethylenethiourea itself was non-toxic. At the lower end of the series this injury consisted of varying degrees of marginal chlorosis and necrosis depending on the homologue and its dosage. This type of injury was most severe, and of about equal intensity, with N-*n*-butyl- and N-*n*-amyl-ethylenethiourea. Both compounds caused an equal amount of necrosis but there was slightly less chlorosis in the plants sprayed with the amyl homologue. The age of the foliage injured depended on the dosage. At 250 p.p.m. only the foliage exposed at the time of spraying was injured but at the higher dosages, injury was observed on foliage developed after spraying. Injury was observed with N-ethylethylenethiourea at all dosages but was restricted to the tips of the leaves. At 250 and 500 p.p.m., no injury was apparent with the hexyl and heptyl homologues. At the two higher dosages, hexyl was the more toxic.

The second type of injury caused by the homologues from octyl to dodecyl consisted of varying degrees of necrotic flecking. This flecking was usually more severe at the tips and margins of the leaves but was not entirely restricted to these areas. It was often evenly distributed over the leaf. The necrotic areas varied in size from 1 to 5 mm. depending on the severity of the injury, which increased as the series ascended from the octyl to the dodecyl homologue. At the octyl homologue it consisted of scattered necrotic flecks while at the dodecyl the necrotic flecks coalesced to form larger necrotic areas with considerable chlorosis around their margins. Injury by these higher members of the series seemed to be restricted to the foliage exposed at the time of application of the chemicals. The homologous series exhibits a peak for one type of phytotoxicity at the butyl or amyl homologue. Another type of phytotoxicity increases as the series ascends from the octyl to the dodecyl homologue.

The toxicity of the N-*n*-alkylethylenethioureas to plant protoplasts was further examined using tissue slices. The procedure developed by Tribe (21) was employed for this purpose. This method, which consists of placing thin disks of tissue in the toxicants and observing their effect on the protoplasts using a plasmolyzing agent containing the vital stain neutral red eliminates many of the barriers with which substances come in contact or pass through to reach the protoplasts of intact plants.

Cylinders were removed from a potato tuber using a cork borer. These cylinders were sectioned into disks of 0.5 mm. thickness with a hand microtome After being washed in running tap water for 24 hr., disks were transferred to 60 mm. Petri dishes containing 5 ml. of the solution under test. Aeration was maintained by continuous gentle agitation. After a suitable length

of time the disks were transferred to a mixture of the following composition: molar potassium nitrate, 8.5 ml.; 0.1% neutral red, 1.0 ml.; phosphate buffer (pH 7.6), a few drops. This was freshly prepared for each estimation as a precipitate was deposited from the solution at the weakly alkaline reaction, which, according to Tribe, is optimal for absorption of the dye by the cells. The disks remained in this solution for 20 minutes and were then transferred to molar potassium nitrate. Comparisons were made visually and the results were recorded by number, thus: 5, whole disk covered with red spots; 4, 3, 2, 1 graduations; 0.5, 0.1, occasional spots; 0, no spots visible. Five disks were used for each determination. The results from the five disks were summed and this total referred to as the Phytotoxicity Index.

In Experiment 1, 20 disks, with a diameter of 7 mm. (No. 3 cork borer) were placed in each solution and at intervals of 12, 24, 36, and 48 hours five disks were removed and examined for kill. Checks consisted of 20 disks in glass-distilled water. The results are given in Table XVII. In Experiment 2, disks of a diameter of 1 cm. (No. 5 cork borer) were treated with a geometric dilution series of each compound as shown in Table XVIII. The disks remained in the solutions for 25 hours. Fig. 10 shows a disk from each treatment photographed, on a sheet of ground glass, with transmitted light.

TABLE XVII

PHYTOTOXICITY OF ETHYLENETHIOUREA AND ITS DERIVATIVES TO POTATO PROTOPLASTS IN RELATION TO TIME

Compound	Concentration, p.p.m.	Phytotoxicity Index* at various periods of time, hr.			
		12	24	36	48
Ethylenethiourea	4000	25	23	19	12
	2000	25	22	21	21
N-Ethylethylenethiourea	4000	25	25	25	22
	2000	25	22	4.1	3.5
N-*n*-Butylethylenethiourea	2000	13	0	0	0
	1000	25	21	4.1	0.6
N-*n*-Amylethylenethiourea	1000	0.4	0	0	0
	500	21	13	2.6	0.1
N-*n*-Hexylethylenethiourea	400	0	0	0	0
	200	21	2.5	0	0
N-*n*-Heptylethylenethiourea	100	0.1	0	0	0
	50	23	13	0.1	0
N-*n*-Octylethylenethiourea	50	0	0	0	0
	25	23	14	3.6	0.2
Check		25	25	25	25

*See text (0, complete kill; 25, no kill).

TABLE XVIII

PHYTOTOXICITY OF ETHYLENETHIOUREA AND ITS DERIVATIVES TO POTATO PROTOPLASTS

Compound	Phytotoxicity Index*							
	25†	50	100	200	400	800	1600	3200
Ethylenethiourea	24	24	25	25	24	23	22	18
N-Ethylethylenethiourea	23	25	24	25	25	23	24	24
N-*n*-Butylethylenethiourea	24	23	22	22	18	9	1.3	
N-*n*-Amylethylenethiourea	21	23	25	20	7	0		
N-*n*-Hexylethylenethiourea	25	21	21	10.5	0			
N-*n*-Heptylethylenethiourea	21	11	0					
N-*n*-Octylethylenethiourea	9	0						
Check	24							

*See text (0, complete kill; 25, no kill).
†Dosage (p.p.m.).

The results of these two experiments show that ethylenethiourea and N-ethylethylenethiourea are relatively non-toxic to potato protoplasts. As the series ascends from N-ethylethylenethiourea to N-*n*-octylethylenethiourea the toxicity to potato protoplasts increases with increasing length of *n*-alkyl side chain. These results agree with those of previous experiments which suggested that when the homologues reach the protoplasts, phytotoxicity increases as the series is ascended up to at least the octyl homologue. The results of Experiment 1 show that N-ethylethylenethiourea killed more protoplasts at 2000 p.p.m. than at 4000 p.p.m. Apparently at the higher dosage osmotic concentration influenced the results.

Discussion

A comparative study of fungitoxicity and phytotoxicity was made with an homologous series of N-*n*-alkylethylenethioureas. The members of this series were found to possess characteristic ultraviolet absorption spectra which could be utilized for qualitative and quantitative analyses. An initial study on some of the physical properties of the series was undertaken as a prerequisite to an examination of their behavior in biological systems. Water solubility decreased and oil/water partition coefficient increased with increasing length of carbon side chain as was anticipated. The water solubilities of the decyl and dodecyl derivatives, as indicated in Table I, were too low for reliable ultraviolet analysis in this medium.

Fungitoxicity was found to increase with increasing chain length up to and including the octyl homologue and to decline sharply in the higher members of the series. This relationship held true for a variety of test fungi and was independent of any of the test methods used.

Probit log concentration regression lines, based on spore assays, for N-*n*-butyl-, N-*n*-amyl-, N-*n*-hexyl-, N-*n*-heptyl-, and N-*n*-octyl-ethylenethiourea were parallel, or nearly parallel as indicated in Table II and Fig. 1.

This indicates that these five homologues all have the same mode of action and that an increase in the length of the alkyl side chain merely results in an increase in fungitoxicity. The change of slope encountered with the nonyl derivative does not necessarily mean that the toxic mechanism has been altered (4). From the dosage–response curves of the nonyl derivative with *Monilinia fructicola* and *Alternaria solani* it may be inferred that some factor such as water insolubility limits the availability of the toxicant. When suspensions are employed, as in these experiments, it might be expected that as the chemical is taken up by the spore more would come into solution and hence become available. The exceedingly slow rate of solution of the higher members of the series, however, minimizes this effect.

The cutoff in fungitoxicity encountered in the homologous series of ethylenethioureas is in agreement with the results in other homologous series where toxicity has been found to increase with rise of molecular weight up to a certain homologue beyond which a decrease in toxicity occurs with further increase in molecular weight (5, 22). Ferguson (10) showed that, in structurally non-specific substances (1), the equitoxic concentrations for members of an homologous series usually fall on a straight line if plotted on a logarithmic scale against water solubility. This toxic concentration line is not parallel to a saturation line plotted on the same scale and hence these lines intersect at the position in the series at which the sharp cutoff in toxicity occurs. A plot of these data, on the ethylenethiourea homologues, presented in Fig. 11 shows that it is possible to predict the position of the cutoff in this homologous series and provides further evidence indicating that the cutoff is due to limiting water solubility. The ED_{50} values fall on curved rather than on the straight lines obtained by Ferguson, but the saturation line intersects the toxic concentration line near the octyl homologue where the cutoff in toxicity was observed. The more resistant the organism the earlier in the series the cutoff should occur. Fig. 11 shows the cutoff for *A. solani* before that of *M. fructicola* but the difference in the resistance of the two organisms is not great enough to shift the cutoff to a lower member of the series.

A summary of the relationships between physical properties and fungitoxicity is presented in Fig. 12. The logarithms of the equitoxic molar concentration (ED_{50} values), particularly of the lower homologues, fall on a straight line when plotted against the number of carbon atoms in the side chain and it is apparent that a correlation exists between water solubility, partition coefficient, and fungitoxic concentration. This is in agreement with the findings of other workers (10) who have investigated toxicity in homologous series. A possible explanation for the break in the correlation for the octyl homologue with *M. fructicola* and the heptyl and octyl homologues with *A. solani* is that micelle formation may be reducing the concentration of single molecules available for toxic action. According to Danielli (8), as the number of carbons in an aliphatic side chain increases the ease of formation of micelles also increases. As the series is ascended from the butyl to the nonyl homologue, the water solubility from one member to the

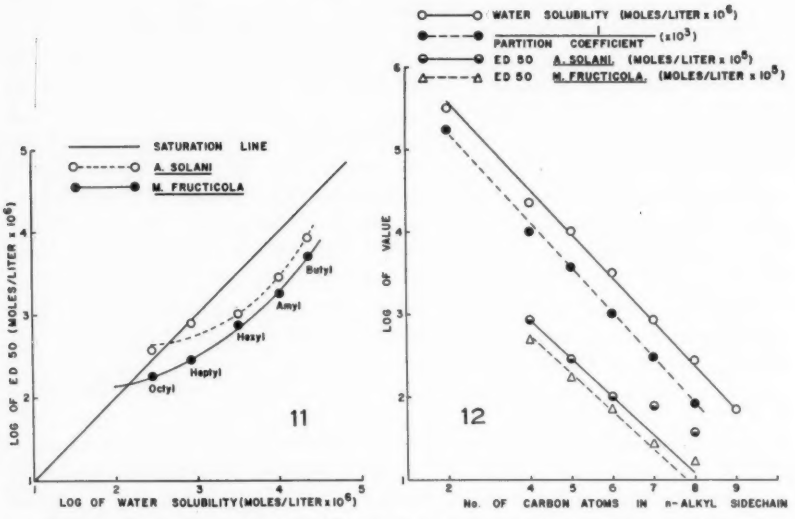


FIG. 11. ED_{50} values for the inhibition of spore germination vs. water solubility for N-*n*-alkylethylenethioureas.

FIG. 12. Properties of N-*n*-alkylethylenethioureas.

next decreases in a geometric progression in which the average ratio is 3.24. The exact opposite relationship holds for the oil/water partition coefficient. From butyl to octyl the coefficients increase in an average ratio of 3.37. That for the toxicity of the homologues to *M. fructicola* and *A. solani* is somewhat lower. The average ratio for the molar concentrations of the ED_{50} values of the homologues from butyl to octyl is 2.37 for *M. fructicola* and 2.25 for *A. solani*. This is in agreement with the results of Stiles and Stirk (13), who found that the average ratios for the surface tensions of eight homologous alcohols was 3.3, whereas that for their toxicities was somewhat lower. On the basis of a molar ratio of 2.37 for the ED_{50} value of *Monilinia* the expected ED_{50} value for the nonyl derivative is approximately 16 p.p.m. This is its saturation solubility in water and is in general agreement with Fig. 2. The expected ED_{50} value for the less sensitive *Alternaria* is 36 p.p.m., more than double the saturation solubility.

Ferguson (10) suggested that, since physical constants, such as water solubility and partition coefficient, are expressions of distribution equilibria, activities, as revealed from thermodynamic potentials, rather than concentrations, should be used as toxic indices. When thermodynamic potentials are not available the ratio of the molar concentration of the toxic solution to its solubility in moles per liter can be used as an approximation. These ratios were calculated for the ethylenethiourea homologues with parallel, or nearly parallel, dosage–response curves.

TABLE XIX

FERGUSON VALUE FOR HOMOLOGUES OF ETHYLENETHIOUREA

Compound	S_t/S_0*					
	Spore germination		Seed germination			
	M. fructicola	*A. solani*	Barley	Wheat	Flax	Radish
N-Ethylethylenethiourea			0.14	0.11	0.15	0.08
N-*n*-Butylethylenethiourea	0.23	0.38	0.42	0.25	0.31	0.15
N-*n*-Amylethylenethiourea	0.16	0.29	0.47	0.38	0.32	0.22
N-*n*-Hexylethylenethiourea	0.24	0.39			0.93	0.77
N-*n*-Heptylethylenethiourea	0.33	0.93				
N-*n*-Octylethylenethiourea	0.64	1.36				

*S_t—ED_{50} (moles/liter).
S_0—Water solubility (moles/liter).

The results given in Table XIX show that, when the proportional saturation of the compounds are compared, the figure remains fairly constant, increasing slightly as the series is ascended, whereas there is a large variation in the corresponding ED_{50} values (Table II). The relatively small change in activity, as indicated by proportional saturation, with increased length of side chain has been noted by Ferguson (10). He suggested that this may be due to chemical constitution. The activity figure varies, for different cells, between 0.01 and 1, the latter being that of a saturated solution (1). A figure of more than 1 was obtained for the N-*n*-octylethylenethiourea with *A. solani*, which means that the ED_{50} was more than a saturated solution. As mentioned previously, the compounds were applied in suspension so the ED_{50} values may not be a true index of the relative amount of chemical presented to the organisms. Nevertheless, they illustrate Ferguson's Principle for this class of compounds. A relatively restricted range of activity values for a group of compounds signifies that their action is mainly physical and determined by phase or adsorption equilibria (10). Values for substances which act chemically vary greatly. Ferguson (10), for example, found those for hydrocyanic acid, ammonia, and monomethylamine to be 0.007, 0.0008, and 0.00023, respectively.

Phytotoxicity as measured by inhibition of seed germination (Fig. 2, Table III) increases as the series is ascended up to the amyl or hexyl homologue, beyond which the compounds are relatively non-toxic. Although the peak of toxicity is at a different homologue, the dosage–response curves for the inhibition of seed germination closely resemble those for the inhibition of germination of fungus spores (Fig. 1). This is particularly apparent with barley and wheat where the slope of the curves remain steep and parallel from ethyl to amyl and then diminish with the hexyl derivative. The results with the heptyl homologue on inhibition of seed germination resemble those

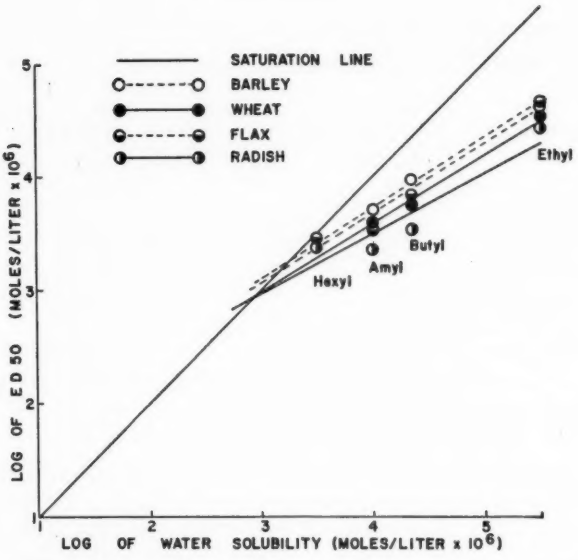


FIG. 13. ED_{50} values for the inhibition of seed germination vs. water solubility for N-*n*-alkylethylenethioureas.

obtained for spore inhibition with the nonyl and higher derivatives in that slight toxicity is exhibited over a wide range of dosages. These similarities suggest that the same property, or properties, in the series is responsible for the cutoff in toxicity to seeds and to spores. The plot of the ED_{50} values and water solubilities given in Fig. 13 shows that, as with fungitoxicity, it is possible to predict the position of the cutoff in phytotoxicity and places this point near the hexyl homologue. The data in Table XIX illustrate that Ferguson's Principle is valid for the phytotoxicity of the ethylenethioureas to seed germination. A peak for phytotoxicity also occurred near the amyl homologue when the ethylenethiourea derivatives were applied to sand in which plants were growing. Two distinct types of phytotoxicity, characterized by different types of injury, occurred, however, when the chemicals were sprayed on the surface of tomato plants. The homologues from ethyl to heptyl caused a general marginal chlorosis with a peak of maximum phytotoxicity at the butyl or amyl homologue. Beginning at octyl and increasing with chain length to the dodecyl homologue the injury consisted of a necrotic flecking. These results indicate that the lower homologues are more mobile in the plant and become evenly distributed throughout the leaf while the higher members of the series remain localized at their point of application. The data presented in Tables IV and V on the translocation of the compounds through tomato plants indicate that as the series is

descended the members become more mobile. The higher members of the series are absorbed by the plant but accumulate in the root system. As the series is ascended the homologues are more readily adsorbed on filter paper so a plausible explanation for the immobility of the higher members is that they are readily adsorbed on such structures as the cell walls, middle lamellae, etc.

The results with excised tomato plants, given in Fig. 9, show that the octyl derivative was more toxic than the ethyl or butyl homologues to the part of the plant to which it was readily available, e.g., the adventitious root system. The latter compounds were transported up through the plants and their toxic symptoms were manifest in the foliage.

The effect of translocation and adsorption on the accessibility of plant protoplasts, to the homologues, was further reduced using Tribe's technique (21) in which thin disks of potato tuber tissue were placed in solutions of the compounds for a suitable length of time and phytotoxicity recorded by observing the number of surviving protoplasts. The results in Tables XVII and XVIII and in Fig. 10 show that toxicity increases as the series is ascended up to the octyl homologue. Since it was not feasible to test the homologues beyond octyl, it is not known whether a cutoff in phytotoxicity to the protoplasts would occur at this homologue.

Thus, for this series of compounds, the results suggest that the accessibility of the cells to the various homologues determines the difference between phytotoxicity and fungitoxicity. As the series is ascended the homologues become less mobile in the plants and it is suggested that their mobility is inversely related to adsorption since the affinity for such surfaces as filter paper increases as the series is ascended. In seeds the higher homologues may be adsorbed on the seed coat or storage tissues before they reach their site of action or even on the glass or filter paper used in the assays. Similarly, when the compounds are applied to sand in which the plants are growing, the higher homologues may be adsorbed by the sand or cellulose cell walls, pectin, suberin, etc. in the root system. The lower homologues are not adsorbed to any extent on these structures and are free to move up the plant.

The results of experiments on the uptake from aqueous solutions of ethylenethiourea and its derivatives, presented in Tables VI and XIII and in Figs. 6, 7, and 8 show that the affinity of the compounds for potato tissue, cellulose, yeast, and *Monilinia* spores increases with increasing length of alkyl side chain. As the series is ascended from one homologue to the next, the uptake by potato tissue and cellulose increases in a geometric progression, whereas the uptake by yeast is in an arithmetic progression. The reason for this difference in the proportion of uptake by yeast and by potato tissue and cellulose is not clear. Uptake in a geometric progression suggests a physical mechanism, and uptake by cellulose would be adsorption. It may be that a different process determines the uptake by yeast. These uptake ratios

were determined on a weight rather than a molar basis but the difference between the two bases from one homologue to the next would be within the experimental error.

Differential partitioning of the compounds (Table XII and XV) is responsible for the uptake gradients in the homologous series by potato tissue and *Monilinia* spores. The proportion of chemical in the biophase, i.e., the potato tissue or spores, increases and the proportion in the external aqueous solution decreases with increasing length of side chain up to at least the octyl homologue. Toxicity in the homologous series also increases with increasing length of side chain. For a toxicant to act it must be available at a toxic concentration in or on the cell. The dosage–response curves of the ethylenethioureas show that they all have the same mode of action so that the distribution of the chemicals between the biophase and the circumambient solution seems to be responsible for their differential toxicity. As the length of side chain increases, the water solubility decreases and a homologue is reached where water insolubility becomes the limiting factor. The ethylenethioureas must be in solution before a partition can occur between the biophase and surrounding medium so the amount of chemical available to the cell is limited by the amount entering the water phase.

No evidence suggesting that the differences in the toxicity of the various homologues is dependent on their ability to penetrate cytoplasmic membranes was obtained in these studies. A correlation exists between their oil/water partition coefficient and their toxicity, but this same correlation exists with water solubility and could probably be demonstrated for other physical factors. If the compounds enter the cells and act on the interior, their differences in lipoid solubility could be an important factor in their accumulation in the cells. Rich and Horsfall (17) postulated that ethylenethiourea might exert fungitoxicity if it were able to reach its site of action within the spore. In this study the data on distribution coefficients indicate that their postulation is not feasible. The biophase/water partition coefficient of ethylenethiourea is very low so that this chemical would always tend to accumulate in the external or water phase rather than at its site of action in or on the spore. The results in Tables VII to X inclusive show that the uptake of the various homologues by potato tissue is not influenced by such factors as salts, pH, temperature, or oxygen, which suggests that uptake is not a function of the permeability of the plasma membrane. If uptake by spores is governed by the same mechanism as uptake by potato tissue, then it can be concluded that, for this series of compounds, toxicity is independent of the permeability of the cytoplasmic membrane.

It is obvious that in this group of compounds there is no selectivity for plant and fungus protoplasm. For example, the ED_{50} value of N-*n*-octylethylenethiourea for *M. fructicola* is 38 p.p.m. (Table II) and that for potato protoplasts would be less than 25 p.p.m. (Fig. 10) so it seems highly improbable that this compound could kill a fungus within a host cell without also killing the cell itself.

Acknowledgments

The authors gratefully acknowledge the assistance and advice given by Dr. H. Martin, Director, Science Service Laboratory, London, during the course of this work and in the preparation of the manuscript. Grateful appreciation is also extended to Dr. G. D. Thorn for his advice on many occasions and to Mr. C. H. Unwin for drawing the graphs.

References

1. ALBERT, A. Selective toxicity. Methuen & Co. Ltd., London. 1951.
2. AMERICAN PHYTOPATHOLOGICAL SOCIETY, COMMITTEE ON STANDARDIZATION OF FUNGICIDAL TESTS. The slide-germination method of evaluating protectant fungicides. Phytopathology, **33**, 627–632 (1943).
3. AMERICAN PHYTOPATHOLOGICAL SOCIETY, COMMITTEE ON STANDARDIZATION OF FUNGICIDAL TESTS. Test tube dilution technique for use with the slide-germination method of evaluating protectant fungicides. Phytopathology, **37**, 354–356 (1947).
4. BARRATT, R. W. and HORSFALL, J. G. Fungicidal action of metallic alkyl bisdithiocarbamates. Connecticut Agr. Expt. Sta. Bull. **508** (1947).
5. BROWN, F. C., BRADSHER, C. E., and LAWTON, E. N. Mildew-preventing activity of rhodanine derivatives. Ind. Eng. Chem. **45**, 1027–1030 (1953).
6. CLARK, A. J. Mode of action of drugs on cells. Edward Arnold & Co., London. 1933.
7. CLARK, W. M. The determination of hydrogen ions. The Williams & Wilkins Co., Baltimore. 1928.
8. DANIELLI, J. F. Cell physiology and pharmacology. Elsevier Pub. Co., Inc., New York. 1950.
9. DAVSON, H. and DANIELLI, J. F. The permeability of natural membranes. Cambridge University Press, London. 1943.
10. FERGUSON, J. The use of chemical potentials as indices of toxicity. Proc. Roy. Soc. B, **127**, 387–404 (1939).
11. HORSFALL, J. G. Principles of fungicidal action. Chronica Botanica Co., Waltham. 1956.
12. LUDWIG, R. A., THORN, G. D., and MILLER, D. M. Studies on the mechanism of fungicidal action of disodium ethylenebisdithiocarbamate (Nabam). Can. J. Botany, **32**, 48–54 (1954).
13. MARTIN, H. The scientific principles of plant protection. Edward Arnold & Co., London. 1940.
14. MILLER, P. M. V-8 juice agar as a general-purpose medium for fungi and bacteria. Phytopathology, **45**, 461–462 (1955).
15. RICH, S. and HORSFALL, J. G. Using dosage–response data to distinguish inherent toxicity from permeation. Abstracts of papers, XII Intern. Congr. Pure and Appl. Chem. 490 (1951).
16. RICH, S. and HORSFALL, J. G. The relation between fungitoxicity, permeation, and lipid solubility. Phytopathology, **42**, 457–460 (1952).
17. RICH, S. and HORSFALL, J. G. Fungitoxicity of ethylenethiourea derivatives. Science, **120**, 122–123 (1954).
18. SEXTON, W. A. The organic chemists approach to chemotherapy. Symposia of the Soc. for Exptl. Biol. No. III. Selective toxicity and antibiotics. Cambridge University Press, London. 1949.
19. SLYKHUIS, J. T. Wheat streak mosaic in Alberta and factors related to its spread. Can. J. Agr. Sci. **33**, 195–197 (1953).
20. THORN, G. D. 1-Alkyl-2-imidazolidinethiones. Can. J. Chem. **33**, 1278–1280 (1955).
21. TRIBE, H. T. Studies in the physiology of parasitism. XIX. On the killing of plant cells by enzymes from *Botrytis cinerea* and *Bacterium aroideae*. Ann. Botany, **19**, 351–368 (1955).
22. WELLMAN, R. H. and MCCALLAN, S. E. A. Glyoxalidine derivatives as foliage fungicides. I. Laboratory studies. Contribs. Boyce Thompson Inst. **14**, 151–160 (1946).
23. WILCOXON, F. and LITCHFIELD, J. T. A simplified method of evaluating dose-effect experiments. American Cyanamid Co., Stamford Research Laboratories, Stamford, Conn.

STUDIES ON LEPTOSPHAERIA AVENARIA f. sp. AVENARIA[1]

DOROTHY E. SHAW[2]

Abstract

Pycnidiospores assignable to *Septoria avenae* Frank and asci and ascospores assignable to *Leptosphaeria avenaria* Weber were isolated from cereals and grasses in Canada, but studies herein reported concern only oat-infecting isolates. Macrospores of the oat-infecting organism occurred on green and mature oats during the summer, on oat stubble in the early autumn, and occasionally on old stubble in spring. Microspores were found on mature oats and stubble at the end of August; they occurred abundantly on stubble throughout the winter, and were still present on a few collections in May and June. Immature perithecia were found on stubble in April and perithecia with mature ascospores on June 16, but stubble moistened in the laboratory before that date had discharged mature ascospores.

Cultures derived from macro-, micro-, and asco-spores produced macro- and micro-spores in culture; of the 82 cultures derived from single ascospores all except seven formed pycnidia and macrospores, and of the 100 cultures derived from single microspores, all except four formed macrospores. No perithecia were found in culture on various media under different conditions, or when isolates were paired in various combinations. The macrospores and ascospores have one nucleus per cell, the nuclei of each spore originating from one nucleus. Microspores are uninucleate.

Newly discharged ascospores germinated in about 1 hour and macrospores in a few hours in water. Microspores took at least 40 hours to germinate on solid media.

In culture, low temperature favored microspore formation and high temperature macrospore formation. Microspores were also found in cultures of *Septoria nodorum* and *S. oudemansii*, but not in cultures of *L. avenaria* f. sp. *triticea*.

The means of overwintering of the organism are discussed; it is considered that, although macro- and micro-spores are available in the spring, ascospores are probably the principal spring inoculum.

Oat-infecting isolates did not infect the other cereals or certain grasses, and the oat-infecting isolates were obtained only from oats. The International Rules of Botanical Nomenclature are interpreted to mean that with the erection of the f. sp. *triticea*, the type automatically becomes *L. avenaria* f. sp. *avenaria* (imperfect *S. avenae* f. sp. *avenae*).

Introduction

The disease of oats caused by *Leptosphaeria avenaria* Weber has assumed increasing importance in Eastern Canada where culm infections has been so heavy that crop lodging has occurred. Moderately heavy infections have been recorded in Agassiz, B.C., but only traces or slight infection in the Prairie Provinces.

In September 1953, diseased oat stubble was collected from the field at Ottawa, Ont. Pycnidia with macrospores were present but, in addition, there occurred on both nodes and leaf remnants smaller pycnidia containing microspores, which had not previously been found in nature, in Canada. This discovery initiated studies on the germination of the microspores, factors influencing their production in culture and in the field, their nuclear condition,

[1]Manuscript received October 19, 1956.

[2]Thomas Lawrance Pawlett Scholar of the University of Sydney at the Science Service Laboratory, Winnipeg, Canada. Present address: Pathologist, Department of Agriculture, Stock and Fisheries, Port Moresby, Papua-New Guinea.

and other aspects. Johnson (6) had recorded microspores in culture and had shown that cultures established from them produced macrospores. Later, Huffman (3) recorded microspores in the field in Iowa in the first week in April and, by the use of spore traps, found them to be a source of primary inoculum for the initial leaf lesions, all indications being that they were principally wind-borne.

"*Septoria avenae* Frank" has been recorded on many graminaceous hosts (Sprague (17), Sprague and Fischer (20)) but since Johnson (5) identified a morphologically-similar organism (*S. avenae* f. sp. *triticea*) on other cereals besides wheat, Sprague (17) suggested that some of the collections on grasses previously assigned to "*S. avenae*" might be *S. avenae* f. sp. *triticea*. Studies were undertaken, therefore, to determine whether isolates from grasses were pathogenic on oats.

Weber (23) recorded the perfect stage (*Leptosphaeria avenaria*) of the oat-infecting *S. avenae* in culture in 1921. Drayton and Bisby (1) and Meehan and Murphy (9) recorded *S. avenae* and an associated *Leptosphaeria* on oats in Canada and Iowa respectively, and Huffman (3) found that perithecia occurred in the field about the first week in June. Since 1921, the perfect stage has not been recorded in culture. Johnson (5) found that *S. avenae* f. sp. *triticea* produced perithecia assignable to *Leptosphaeria avenaria* in culture, as well as on wheat in the field. The studies here reported were undertaken to determine the occurrence of the perfect stage in the field and in culture, and to determine whether isolates of "*Leptosphaeria avenaria*" from cereals and grasses were pathogenic on oats.

Materials and Methods

Pycnidiospores assignable to *S. avenae* Frank and asci and ascospores assignable to *L. avenaria* Weber were isolated from wheat, oats, barley, rye, and grasses in Canada. For comparative purposes, pycnidiospores of *S. nodorum* isolated from wheat from England and Canada were also included in all experiments.

Single pycnidiospore, single ascospore, or single ascus cultures were established from the above and were (1) subcultured by means of single spores, either asexual or sexual, which were isolated on potato-sucrose agar, transferred to potato-sucrose agar slopes and subcultured on various media, particularly sterile citrus leaves, and (2) maintained by mycelial transfer on potato-sucrose agar. Most cultures lost the ability to sporulate when maintained by mycelial transfer.

The isolates were tested on a variety of media, including potato-sucrose agar, sterile citrus leaves, maize grain on cob, maize leaves, asparagus stems, alfalfa stems, wheat grains, oat straw, wheat straw, carrot and potato agar, chopped oat leaves in agar, ground oat hull agar and, following Nitimargi (11), an agar medium with different proportions of carbon and nitrogen source. Johnson (6) found that production of pycnidia was abundant on sterile citrus leaves, and this was confirmed.

Inoculation tests were carried out on 'Victory' and/or 'Red Rustproof' oats, 'Gaza' wheat, 'Vantage' barley, and 'Prolific' and/or 'Rosen' rye. The grasses used in the tests were established from seed supplied by the Botany Unit, Ottawa, or from the field, in which case the identity was checked by Dr. D. Löve, Department of Botany, University of Manitoba, or they originated from clones from established plants at the Science Service Laboratory, Winnipeg. The inoculum was either spores from the field or culture, which were sprayed on the plants, or macerated mycelium, which was applied to the leaves with an inoculating scalpel. Unless stated otherwise, plants were incubated for 48 hours.

This study would not have been possible without the assistance of collectors in Eastern Canada who forwarded oat plants and stubble from the field throughout five seasons for periodical examination at Winnipeg. Also received periodically were samples of oat stubble which Mr. R. A. Derick, Cereal Crops Division, Ottawa, had lifted from the soil at the end of summer and placed in flats at Ottawa. This stubble was left without protection in the snow under as near natural conditions as possible.

Pathogenicity of Isolates

The isolates from cereals and grasses fell into two groups: (1) those which were nonpathogenic on oats and (2) those which were pathogenic on oats.

The collections from which the isolates of the first group were obtained were mainly of senescent leaves and sheaths or stubble. They are considered to be distinct from the oat-infecting *L. avenaria*, and are discussed elsewhere (15).

The Occurrence of the Spore Forms of the Oat-infecting *L. avenaria* on Field Material and in Culture

Standing, diseased oat stubble with 6–8 in. of the stem remaining and with the "black stem" symptom on many stems was examined in Ottawa in September 1953. In many cases the second node from the base was covered with pycnidia in a manner reminiscent of *S. nodorum* (Fig. 1). The pycnidia contained either macrospores, macrospores and what was tentatively taken to be microspores, or solely microspores. Some of the expressed contents of a pycnidium containing both spore forms are shown in Fig. 3. Some intermediate-sized spores were present in pycnidia with the two spore forms, but these were always greatly in the minority. Such intermediate types are shown in Fig. 3. The microspores were single-celled, hyaline, rod-shaped with round ends, slightly curved, measured 2.5–5 $\times$ 1 μ and agreed well with those described by Johnson. Germination of the microspores was not obtained at the time because, as shown later, the tests were concluded too soon. The connection with the macrospores could therefore not be established. On the circumstantial evidence of having been found in the same pycnidium, however, the small spores were thought to be microspores of the oat-infecting *S. avenae*.

In April and May, 1954, cultures established from macrospores from collections from five localities in Eastern and Western Canada, which had been paired in all possible combinations on sterile asparagus stems in an attempt to produce the perfect stage, were found to be producing pycnidia with microspores. As the microspores also occurred in some of the unpaired controls, it is considered that the pairing was not responsible for their production. These microspores also agreed well with those described by Johnson.

Thirty-two single-microspore cultures were established from these microspores on potato-sucrose agar and then subcultured after about 6 days on sterile citrus leaves. Pycnidia and macropycnidiospores formed abundantly on this substrate and to a less extent on potato-sucrose agar. One culture produced both macro- and micro-spores. Mycelium and macrospores from the cultures were inoculated to 'Red Rustproof' oats, and lesions similar to those produced by the macrospores from the field appeared in about 4 days. Some of the lesions were surface-sterilized and plated on potato-sucrose agar, and typical colonies of the oat-infecting *S. avenae* were recovered. After about 10 days the oat plants were covered with glass chimneys in order to maintain a high humidity, and pycnidia and macrospores were later found in many lesions. As the work progressed, microspores were found in cultures of other isolates.

Because the microspores within the pycnidium could be confused with species of *Phoma* and *Phyllosticta* and, when free of the pycnidium, with other single-celled, hyaline spores of many species, and because the pathogenicity of these and the other spore forms was in question, single spores of all the forms were isolated from as many collections as possible, and the cultures established from them checked for pathogenicity and production of spore forms. Only those cultures which were handled in this way are shown in Table I. The results of the study of the collections are as follows:

TABLE I

NUMBER OF FIELD COLLECTIONS STUDIED, THE OCCURRENCE OF THE SPORE FORMS, AND NUMBER OF SINGLE SPORE ISOLATES OF OAT-INFECTING *L. avenaria* THAT PRODUCED MACROSPORES IN CULTURE

Cultures from:	No. of field collections represented	Occurrence	No. of single-spore isolates	No. of isolates producing macrospores in culture*
Macrospores	17	Summer† Autumn Spring	66	58
Microspores	12	Autumn Winter† Spring	100	96
Ascospores	10	Spring†	82	75

*Random isolates of seven, five, and two collections tested, respectively, all produced microspores in culture when held at low temperature.
†Abundant.

The pycnidia which occurred in lesions on green leaves, that is, on growing oat plants, contained macrospores only.

In Eastern Canada, microspores occurred on mature oat plants at the end of the growing season in 1953 and 1954, as early as the end of August. In Iowa, Huffman (3) found that they occurred on stubble in April, *after* the winter. In the Canadian collections examined, pycnidia with microspores were intermingled with pycnidia with macrospores. Occasionally both spore forms were found in the same pycnidium.

Oat stubble which remained in the field throughout the winter, in some cases under three to four feet of snow, and which was examined periodically, contained pycnidia with only microspores. The micropycnidia were found on sheath remnants, scattered over the nodes, or arranged along the internodes in lines corresponding to the chlorenchyma. These pycnidia were sometimes as long as 180 μ. Macropycnidia were not detected on the stubble examined throughout the winter. A few collections from the field in May and June still contained pycnidia with microspores, although they were not found in the majority of the samples.

In May and June some collections of stubble were found to contain pycnidia with macrospores. Whether these pycnidia were newly formed in the spring, as the temperature rose, or whether they had contained microspores throughout the winter, and gave rise to macrospores only when the microspores had been exuded and the temperature rose in the spring, is not known.

Oats from Agassiz, B.C., were found to have immature perithecia in April. Immature asci, paraphyses, and ascogenous hyphae were pressed out of perithecia, and growth was obtained in two cases. This mycelium produced colonies resembling the oat-infecting *S. avenae* and typical macrospores, and the mycelium was vigorously pathogenic on oats. Because it is not definitely known that the mycelium came from the contents of the perithecia it cannot be said that the genetical connection was demonstrated. However, oat-infecting ascospores of *Leptosphaeria* were later discharged from this stubble after it was wetted and dried in the laboratory. The climate at Agassiz is milder than in Eastern Canada, and it may be that the perfect stage forms there earlier than in the east.

Immature perithecia were found on collections from Eastern Canada in April, and even stubble collected as early as March and kept in the laboratory was capable of producing perithecia with viable ascospores, after it was wetted and dried, in June. The first collection from the field to have mature ascospores when examined was that of June 16. However, as viable ascospores had been discharged from stubble kept in a humid atmosphere in the laboratory before this, and since wetting could have occurred in the field before this also, it is probable that ascospores were being discharged from stubble in Eastern Canada early in June or even in May. This is in accord with the observation of Huffman (3) that perithecia occurred about the first week in June in Iowa.

Of the 66 cultures derived from macrospores, all except eight (from four collections) produced macrospores. Of the 82 cultures derived from single

ascospores, all except seven (from three collections) formed pycnidia and macrospores. Of the 100 cultures derived from single microspores, all except four (from two collections) formed macrospores. Occasionally microspores were found in cultures held at room temperature, or in cultures kept in a greenhouse annex where fluctuations in temperature were possible. All cultures which were placed at low temperatures (random isolates from 14 collections) formed microspores if held there for longer than 3 weeks, whether they were derived from macro-, micro- or asco-spores. Cultures in which microspores are not recorded might have been capable of producing them if they had been held at low temperatures. The lack of perithecia in culture is referred to in a later section.

The majority of the isolates had colonies which, although not exactly identical, could be termed 'wild type'. The colonies were white at first, then slightly buff, compact, grew more slowly on potato-sucrose agar than cultures of *L. avenaria* f. sp. *triticea*, and contained a deep-brown pigment in the medium. Some of the cultures differed slightly from the 'wild type', mainly in growth rate, color of mycelium, and depth of pigment.

The Perfect Stage in Culture

Weber (23) obtained perithecia in 4-month-old cultures derived from pycnidiospores from diseased oats, and in 6-week-old cultures derived from single ascospores. The ascospores were pathogenic on oats, and on the basis of these results Weber described *Leptosphaeria avenaria* sp. nov. To the writer's knowledge, this is the only report of the perfect stage in culture.

In this study no perithecia developed in any culture of the oat-infecting *S. avenae* on any of the media listed under "Materials and Methods". The cultures were maintained under various conditions of light and temperature. To test for heterothallism in the oat form the isolates, from seven localities in Eastern Canada and from British Columbia, were paired in all combinations, but no perithecia were obtained.

When cultures were established from single ascospores from the field, eight were taken at random from one collection from Ottawa and mated in various combinations on sterile citrus leaves, which were the best medium for perithecial formation of *L. avenaria* f. sp. *triticea*. One set was held at room temperature; another set was kept at 6–7° C. for 3 weeks and then

---

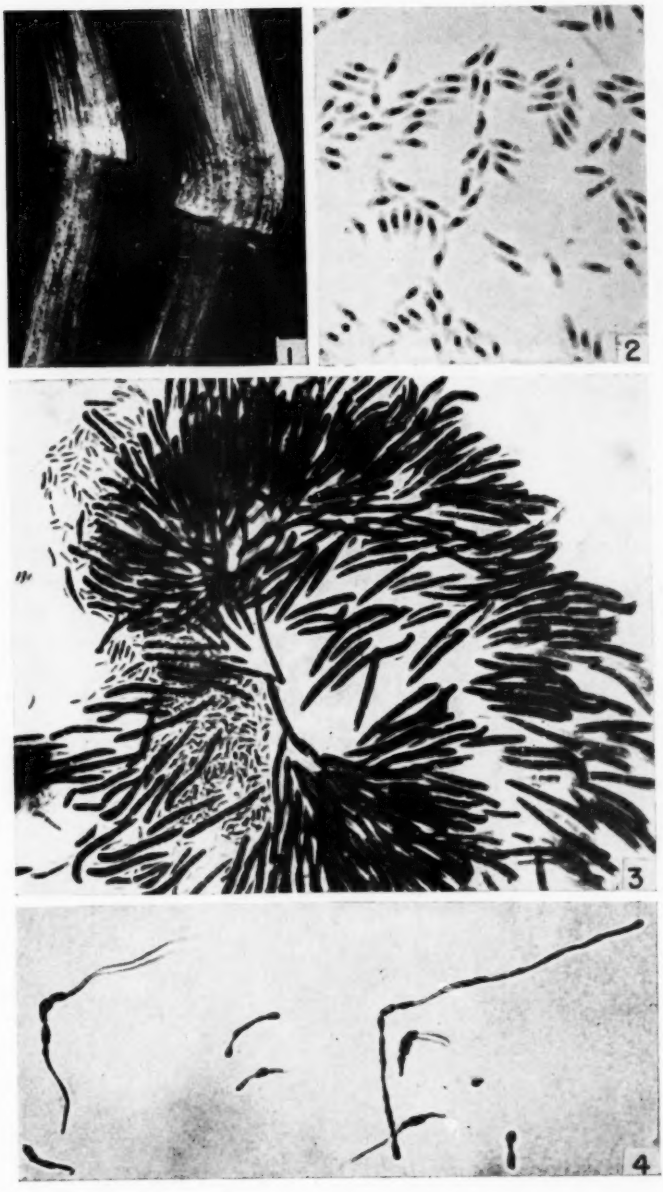
FIG. 1. Portions of oat stems which had remained in the field until Jan. 17 with pycnidia of the oat-infecting *L. avenaria* on the nodes and internodes. Microspores were contained in the pycnidia. ×4

FIG. 2. Microspores of the oat-infecting *L. avenaria* from nature, showing one nucleus per spore. Stained with Giemsa after acid hydrolysis. ×1200

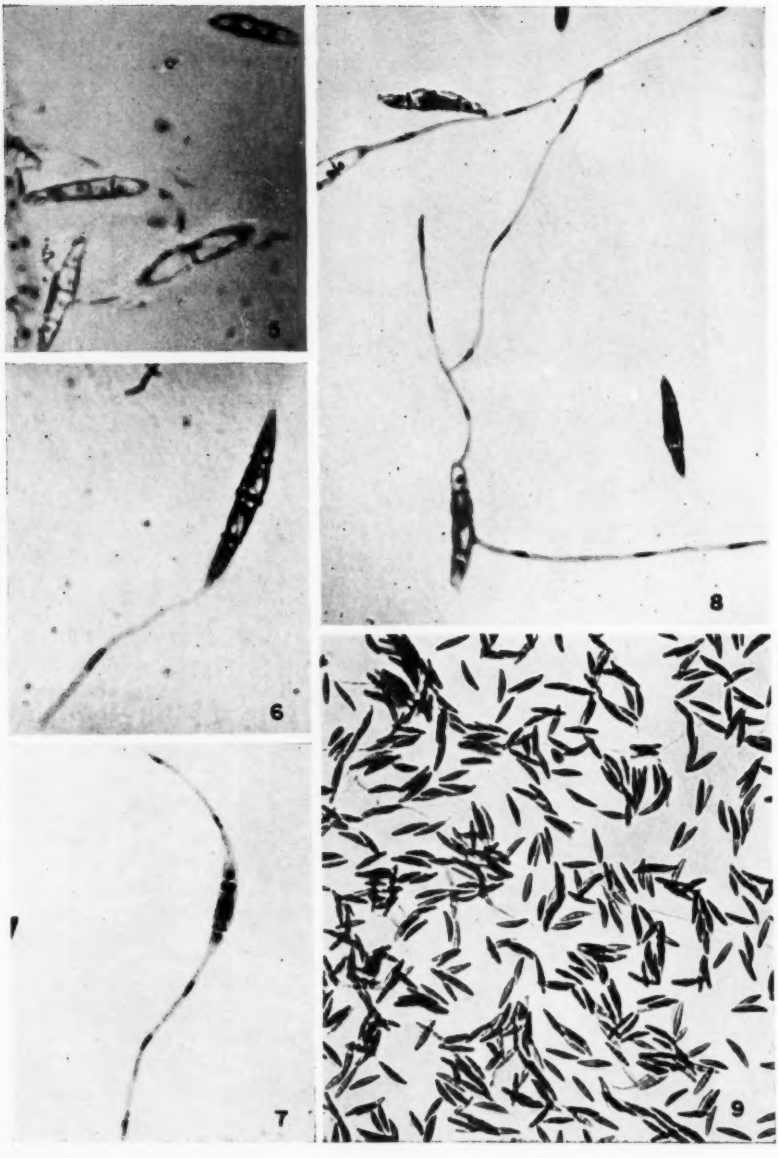
FIG. 3. Some of the contents of a pycnidium of the oat-infecting *L. avenaria* from a node of stubble from the field on Sept. 9, showing macrospores (3-septate), a few intermediate spores (0–1-septate), and bacteroid microspores (0-septate). Mounted in cotton blue lactophenol. ×350

FIG. 4. Germinating, unstained microspores of the oat-infecting *L. avenaria* on potato-sucrose agar after 48 hours. ×350

PLATE I



Shaw—Can. J. Botany


5
6
7
8
9

transferred to room temperature for 5 weeks, at which time pycnidia with macrospores only were produced.[3] A third set was held at 6–7° C. for 8 weeks at which time pycnidia with microspores only were produced.

No perithecia were observed in any of these cultures, and Johnson (6) did not obtain perithecia when he added microspores to cultures of the oat-infecting *S. avenae*, in an attempt to determine whether the microspores acted as spermatia.

Shaw (15) found that *L. avenaria* f. sp. *triticea* was homothallic. Its life cycle, however, is different from that of the oat-infecting organism. No microspores have been recorded for the f. sp. *triticea*. Its perithecia, with mature ascospores, have been recorded on mature plants and stubble in the autumn and on stubble in the spring in Canada (15) and on mature plants in the late spring and on stubble in the winter in Australia (13). Its perfect stage, therefore, seems to form more readily and under a wider range of conditions than does the perfect stage of the oat-infecting organism; in the latter overwintering appears to be necessary for the formation of perithecia.

The lack of perithecia in culture in the present study might be due not to the inherent inability of the isolates to produce them but to the fact that the media used were unsatisfactory for the production of the perfect stage, or that the right environment was not provided.

The fact that perithecia succeeded microspores on the stubble does not indicate that the microspores acted as spermatia; they could be merely asexual spores with no function in the formation of the sexual stage. The fruit bodies examined at what might be called the transition period were either pycnidia with microspores or immature perithecia with paraphyses. A more detailed examination of the fruit bodies at the transition period is required.

Dissemination, Germination, and Infectivity of the Spores

The macrospores are typical slime spores and are exuded from the pycnidium in water. If leaves are maintained in a humid atmosphere the spores exude

[3]Later work showed that the period of 3 weeks was not long enough to allow the production of microspores at the low temperature.

FIG. 5. Immature ascospores of *L. avenaria* f. sp. *triticea* showing 1-septate spore with two nuclei, 1-septate spore with four nuclei, and 3-septate spores with four nuclei each. Stained with Giemsa after acid hydrolysis. ×800

FIG. 6. Germinating ascospore of *L. avenaria* f. sp. *triticea* showing one nucleus in the germ tube and one nucleus per cell in the spore. Stained with Giemsa after acid hydrolysis. ×1100

FIG. 7. Germinating ascospore of the oat-infecting *L. avenaria* showing regularly-spaced nuclei in the germ tube. Stained with Giemsa after acid hydrolysis. ×600

FIG. 8. Mature and germinating ascospores of the oat-infecting *L. avenaria* showing a later stage with regularly-spaced nuclei in the germ tubes with no clustering at the tips, and one nucleus per cell in the spores. Stained with Giemsa after acid hydrolysis. ×800

FIG. 9. Unstained ascospores of the oat-infecting *L. avenaria* discharged overnight from overwintered straw suspended in a humid atmosphere on a glass slide. Note that some ascospores have germinated. ×200

as a glistening mass spread around the ostiole. If the leaves are kept in a humid atmosphere with the ends in water, however, the spores exude in long cirri. The exuded spores in the mass or in the cirri can then be disseminated by water.

Information was sought as to the viability of spores exuded on leaf surfaces and exposed to sunlight. Heavily diseased oat leaves were kept in a humid atmosphere with the leaf bases in water for 14 hours until the spores had exuded from the pycnidia in long cirri. The leaves were then placed in dry Petri dishes to protect them from the rain and left in the open outside the laboratory in intermittent sunshine. Immediately, and every week for 4 weeks, three or four cirri were lifted, with needles, from the leaves in each dish, dispersed in water, streaked on potato-sucrose agar, and examined for the presence of germ tubes in 6 hours. After 4 weeks, 97% germination was recorded.

In another experiment leaves with exuded cirri were placed parallel to streams of air at 5–7, 12–14, and 18–20 m.p.h. as measured with an anemometer. A greased glass plate was held perpendicular to the air stream immediately behind the leaves. No cirri or spores were blown onto the plate, although talc, wheat stem rust, and powdery mildew spores were deposited in separate tests. In the field, of course, exuded cirri might be dislodged by insects and other agents and could then be blown by the wind.

Macrospores from the field and from culture germinate in water or on an agar surface in a few hours and in this time 100% germination can be obtained with macrospores from recently collected material. Infection of oats was obtained when seedlings, sprayed with a water suspension of macrospores, were incubated in a humidity chamber overnight. An incubation of 48 hours was usually maintained, however, to ensure a good development of lesions.

When germination tests were conducted with microspores from the 1953 collections, it was considered that they were inviable. Later work as detailed below showed that the tests, which were made at the same time as tests with macrospores, had been terminated too soon.

The microspores germinate very slowly on potato-sucrose and plain agar, swelling first until oval in shape, then producing a germ tube at one end, usually followed by another germ tube from the other end. Germination does not begin until after about 40 hours, and additional spores proceed to germinate during the next few days. The times of germination of microspores from field material which was kept in the laboratory at room temperature are given in Table II. Also tested were microspores of Collection No. 172, a portion of which was hung outside the laboratory under natural conditions, and macrospores from two collections. In the tests 100 macrospores or microspores were examined at random. Again, because of the indistinctive morphology of the microspores, single spores were isolated in every test for the establishment of cultures that were taken through the full procedure of identification and infection of oats.

TABLE II

GERMINATION (%) OF MACRO- AND MICRO-SPORES OF THE OAT-INFECTING *S. avenae* AND THE RESULTS OF CHECKING TESTS

Spores tested	Hours					No. of spores isolated as a check	Reaction on 'Victory' oats of the check cultures	Spore form in the check cultures at room temperature
	6	24	48	72	96			
Macrospores								
from 4 collections P.S.A.	90					3	Susceptible	Macrospores
1 collection water	79							
Microspores								
from 14 collections P.S.A.	0	0	71	86	92	50	Susceptible	Macrospores
2 collections water	0	0	0	0	0			
3 collections other liquids	0	0	0	0	0			

No difference was noted in germination time or behavior of microspores from the field or from culture. Lining the lids of Petri plates with wet filter paper did not accelerate germination of microspores on potato-sucrose agar. No germination of microspores was noted in liquid media such as water, dilute potato juice, and sucrose solution, dilute glucose solution, straw decoction, or soil extract solution. In Fig. 4 unstained microspores are shown germinating on potato-sucrose agar at 48 hours. Some of the spores have long germ tubes; others have just germinated.

Infection of oat plants by microspores was obtained when the plants were maintained in a humid atmosphere sufficiently long to allow germination and penetration. A period of 5 days at a high humidity was found to suffice.

Ascospores that were discharged on an agar surface in a Petri dish, from stubble attached to wet filter paper on the undersurface of the lid, germinated in about 1 hour. Even when the agar surface was replaced by glass slides, some germination occurred on the slides. In Fig. 9, some germ tubes are discernible in ascospores discharged on a glass slide overnight. When the contents of a perithecium were pressed out into water, single ascospores, and ascospores still retained in the asci, germinated in about 1 hour. Infection of oats was obtained when wet straw with discharging ascospores was suspended over oat seedlings in a humidity chamber overnight.

The lesions obtained from ascospore, macrospore, and microspore infections were identical. The time for infection was shortest with ascospores, followed closely by that for macrospores, but much longer with microspores.

Microspores in Other Species

In April 1954, pycnidia with microspores were found in cultures of *S. nodorum* Berk., originally established from single macropycnidiospores from collections from Scott, Sask., Edmonton, Alta., Normandin, Que., and Fredericton, N.B. The isolates had been paired in all combinations on sterile maize grain, in an attempt to produce the perfect stage. As pycnidia and microspores also occurred in some of the unpaired controls it was considered that the pairing was not responsible for their production. Subsequently,

microspores were found in cultures established from single macrospores from wheat from Lilyfield, Man. The microspores were single-celled, hyaline, rod-shaped with round ends, and measured 2.5–5 × 0.8–1 μ.

Thirty-three single microspores were isolated and established on potato-sucrose agar, subcultured on sterile citrus leaves, inoculated to 'Marquis' or 'Little Club' wheat and, in some cases, the resulting lesions were surface-sterilized and plated on potato-sucrose agar. In other cases the inoculated plants were covered with glass chimneys and, later, pycnidia and macrospores were found in some of the lesions. No microspores have previously been recorded for *S. nodorum*.

Pycnidia with microspores were also found in cultures of *S. oudemansii* Sacc., originally established from macrospores from *Poa pratensis*, on sterile citrus leaves. Pycnidia with macrospores were also present. The microspores were hyaline, rod-shaped, and measured 2.5–4 × 0.8–1 μ. Ten single microspores were isolated and established on potato-sucrose agar. These cultures produced abundant macro- and micro-pycnidia. When the cultures were inoculated to wheat and *Poa pratensis*, the former was immune while the latter showed a trace infection.

Frandsen (2) reported microspores in some species of *Septoria* on the Gramineae and proposed that a division of the genus be made on this character. Sprague (17) recorded microspores in eight species of *Septoria* on various grasses. Johnson (6), however, who recorded microspores in cultures of *S. avenae*, obtained germination and proved the connection with the macroform. Dr. Mary Noble notified Dr. T. Johnson by letter that she had found spermatium-like microspores in ground oat hull cultures and oatmeal agar cultures of *S. avenae* in Scotland. Huffman (3) recorded microspores on oats in the field.

Other species of *Septoria* might produce microspores and it is possible that species of *Phoma* and *Phyllosticta* already recorded on the Gramineae might be the microspore stages of species with well-known macrospores of *Septoria*. Therefore, there would probably be no permanency of groups separated on this character, that is, production of microspores, as proposed by Frandsen, until much more information is available on the spore forms of species under various conditions in the field and in culture.

Factors Responsible for Microspore Formation

Johnson (6) obtained results with the oat-infecting *S. avenae* which indicated that low temperature, while favorable to microspore formation, tended to have an inhibitory effect on macrospore formation.

In order to obtain more information on the role of temperature in macro- and micro-spore production, isolates which had been found to be microspore producers, such as the oat-infecting *L. avenaria* (three isolates), *S. nodorum*, and *S. oudemansii* were subcultured on sterile cirtus leaves from 4- to 5-day-old single macrospore cultures on potato-sucrose agar. Also treated in this way were cultures of *L. avenaria* f. sp. *triticea* (three isolates, No. 142 derived

from a pycnidiospore from oats, No. 154 derived from an ascospore from wheat, and No. 157 derived from a pycnidiospore from wheat), and cultures of *Septoria* sp. from *Anthoxanthum odoratum*, *Hendersonia* sp. from wheat, and *Phaeoseptoria poae* Sprague from *Poa compressa*. The cultures were held at 5–6, 12–13, and 21–22° C. and were read four times over a period of 4 months by sampling the fruiting bodies formed and determining the spore forms present. The results are summarized in Table III.

TABLE III

SPORE FORMS PRODUCED BY VARIOUS ISOLATES AT DIFFERENT TEMPERATURES

Acc.	Isolate	5–6° C.	12–13° C.	21–22° C.
88	*S. oudemansii*	—*	*Micro*	Macro
47	*L. avenaria* (oat-infecting)	—*	*Micro*	Macro
133	"	*Micro*	*Micro*	Macro
134	"	*Micro*	*Micro*	*Micro* and Macro
99	*S. nodorum* (wheat-infecting)	—	*Micro*	Macro
80	*Septoria* sp. (from *Anthoxanthum odoratum*)	—	Macro	Macro
142	*L. avenaria* f. sp. *triticea*	—	—	Macro and Ascospores
154	"	—	—	Macro and Ascospores
157	"	—	—	Macro and Ascospores
111	*Hendersonia* sp.	Macro	Macro	Macro
86	*Phaeoseptoria poae*	Macro	Macro	Macro

*At 2° C.

The microspore-producing isolates all had the same tendency: mainly macrospore production at high temperature and mainly microspore production at low. No microspores were produced by the *L. avenaria* f. sp. *triticea* isolates at any of the three temperatures, and asexual and sexual spores were produced only at the highest temperature. The isolates of *Septoria* sp., *Hendersonia* sp., and *Phaeoseptoria poae* produced only macrospores.

This tendency was confirmed repeatedly throughout the work. In another experiment, as reported in the section "The Perfect Stage in Culture" it was found that, as growth at the low temperature is slow, more than 3 weeks is required for the production of micropycnidia in culture. All isolates in this study, which were pathogenic on oats and which were held at the low temperature for a sufficient length of time, produced microspores.

From these tests it was concluded that, on the media used, the macro-form predominated over the micro-form at the higher temperature, while the micro-form predominated at the lower, in those strains capable of dimorphism of asexual spores, that is, the oat-infecting *L. avenaria*, the wheat-infecting *S. nodorum*, and *S. oudemansii*. Variation in temperature failed to induce microspore formation in isolates of *L. avenaria* f. sp. *triticea*.

The fact that microspores were found in the field in Canada at the end of August and in early September, that is, when the temperature is falling, and throughout the winter, and that macrospores were found on stubble in

the spring, when the temperature is rising, and on the summer crop, would seem to indicate that temperature is one of the main factors influencing the production of spore forms in the field as well as in culture.

Temperature may not be the main factor influencing the production of the spore forms in other species of *Septoria*. For example, in Australia, Shaw (13) found that macrospores of *S. tritici*, one of the filiform-spored species, occur abundantly on wheat in the winter, and microspores on the mature plants in the early summer, when temperature is rising, whereas in Canada (unpublished data) macrospores of *S. tritici* occur on wheat in the summer, and microspores on mature plants in the autumn, when temperature is falling. In this case the physiological state of the host may be a more important factor than temperature.

Overwintering of the Organism

In discussing the mode of overwintering of the organism, Weber (23) stated that he had tied detached oat leaves containing pycnidia and macrospores to a stake in a field and tested the viability every 2 weeks. The germination was found to be 90% even 6 months later, on April 1. Stanton (21) reported the occurrence of a kernel blight phase of the oat organism in several northern States in 1952, but Simons and Murphy (16) found that the disease was not transmitted through infected seed. Noble and Montgomerie (12), reporting on the disease in Scotland, stated that recent work at East Craigs showed that the disease is seed-borne. Huffman (3) considered that, in Iowa, the fungus persists in the mycelial form on stubble through the late summer and winter, and that microspores, which were first noted in April, constitute the primary inoculum, the microspores being principally wind-borne.

As reported herein, although macrospores were present on the growing and mature plants in Eastern Canada, microspores were present even at the end of August, particularly on the nodes, and were abundant on nodes and internodes throughout the winter. Immature perithecia were present in the spring, and macrospores were found in May. In late May and early June, therefore, all three spore forms were present. The number of microspores appeared to be decreasing, the number of macrospores to be increasing, and the ascospores were very abundant.

All three spore forms are capable of germination, and have the ability to infect oats, but whereas the ascospores germinate in about 1 hour, and the macrospores in a few hours, the microspores need about 40 hours, and even then only a small proportion have germinated. Conditions favorable for germination and penetration of the leaf surface must be maintained for a much greater length of time for microspores than for ascospores or macrospores.

Also, because ascospores can be discharged from the asci they have a better chance than the slime spores of being carried to leaf surfaces. Both macro- and micro-spores, and asci and ascospores, could be dispersed by water, and could probably be splashed on to leaves.

As mentioned previously, temperature is probably the main factor in the field influencing the production of the two asexual spore forms. The relative

proportion of the macro- and micro-spores in the autumn and spring is probably determined by the temperatures operating at the time. It is not known at present what factors influence the production of the perithecia; the temperature, the maturity of the thalli, the composition of the substrate, and the possibility that microspores act as spermatia will have to be considered. The proportion of ascospores, microspores, and macrospores on stubble in the field may alter from year to year and from region to region depending on the environment.

The Nuclear Condition of the Spore Forms

The nuclear condition of *L. avenaria* (oat-infecting) has not previously been determined, except for the work of Shaw (14), who showed that the macrospores contained one nucleus per cell, so that the number of nuclei per spore equals the number of cells.

In this study two procedures were used to study the nuclear condition:

1. Pycnidia and perithecia were placed directly into 2% aceto-orcein in 60% acetic acid, crushed, warmed, and examined immediately or stored at a low temperature overnight and examined on the following day.

2. Spores were allowed to exude in water on slides, or perithecia were crushed in water and as much of the wall removed as possible, or mycelium was added directly to the water. The water was allowed to evaporate at once, or after certain periods of time in germination studies. The material was hardened with methanol and stained with Giemsa after acid hydrolysis in *N* HCl at 60° C. as given in detail by Shaw (14), except that ethanol was replaced by methanol at the suggestion of Dr. W. Jacobson of the Strangeways Research Laboratory, Cambridge, England, and Canada balsam was used for mounting instead of euparal. According to Murray *et al.* (10), Tulasne and Vendreley (22), and Jacobson and Webb (4), Giemsa demonstrates the distribution of desoxyribonucleic acid.

The macropycnidiospores of the isolates under study were found to have one nucleus per cell, thus confirming the previous report (14). Immature spores which are still unicellular have one nucleus per spore.

It was found that when the macrospore begins to germinate, the germ tube is at first anucleate. Then the nucleus in the cell of the spore divides; one daughter nucleus appears in the germ tube a short distance from the cell of the spore, and the other daughter nucleus remains in the cell of the spore. If another germ tube issues from the same cell, the latter daughter nucleus divides and supplies the nucleus for the second germ tube.

As the germ tube grows the nuclei in it are spaced at fairly regular intervals along the hyphae. The nuclei never appear clustered at the tip as reported for some other organisms, for example, *Fusarium culmorum* (Jones (7)). In older hyphae, which are strongly septate, there is one nucleus per cell. In hyphal tips the septations are often difficult to see, but the nuclei are still spaced about the same distance apart. The cytoplasm of the tip of germ tubes from spores germinated in water is homogeneous compared with the vacuolated condition of the older part of the germ tube and the spore.

The microspores were found to be uninucleate, as shown in Fig. 2.

The paraphyses, which are multicellular, have one nucleus per cell. The cytoplasm in the paraphyses appears to stain less with aceto-orcein than does the cytoplasm in the spores or the mycelium.

The cell wall of the mature ascospore is pigmented, and difficulty was experienced in staining the nuclei of mature ascospores with aceto-orcein, and even with Giemsa after acid hydrolysis at 60° C. there was some difficulty. However, near-mature spores, as judged by the development of pigment, and spores which have just begun to germinate, have one nucleus per cell. The germ tube is first devoid of a nucleus, then one appears at a little distance from the tip and from the original cell, as shown in Fig. 6 for *L. avenaria* f. sp. *triticea*. As the germ tube lengthens the nuclei in it are spaced at fairly regular intervals along the hyphae, as shown in Figs. 7 and 8 for the oat-infecting *L. avenaria*. They were never found clustered at the tip.

The major nuclear happenings were followed in the asci of *L. avenaria* f. sp. *triticea*, which could be produced at will in culture, from the time of the fusion nucleus in the primary ascus to the maturation of the ascospores. Further work with the oat-infecting organism will reveal whether or not any departures from this procedure occur.

The fusion nucleus is large compared with the nuclei in the mycelium. In the asci with two and four nuclei, the nuclei are arranged along the long axis of the ascus. The spindles of the eight nuclei at a later stage are slightly oblique to the long axis, and the spindles of the 16 nuclei at a still later stage are more oblique again. From the proximity (as shown in the material stained with aceto-orcein) of the two nuclei in each of the eight pairs at the 16-nucleate stage (especially of the pairs at the tip of an ascus) and from the density of the cytoplasm around the pairs, it is concluded that the nuclei of an ascospore are derived from only one of the eight nuclei present after the third division.

At the 16-nucleate stage the oblique ascospores are already delimited, and if the ascus wall is broken at this stage, the immature ascospores separate from each other and from the ascus wall with ease. In Fig. 5, an ascospore with one septum and with one nucleus in each cell can be seen entirely free from the ascus cytoplasm. Each nucleus then divides, as shown in Fig. 5, where a one-septate spore is visible with four nuclei, and a septum forms between the two pairs, so that the near-mature ascospore has one nucleus per cell.

It is concluded that the microspores are uninucleate, and the macrospores and ascospores have initially only one nucleus per cell. Exceptions might occur, but they were not noted in this study.

Host Range and Nomenclature

Sprague (17, 18, 19) and Sprague and Fischer (20) listed *S. avenae* as occurring on species and varieties of *Agrostis*, *Arrhenatherum*, *Calamagrostis*, *Festuca*, *Glyceria*, *Oryzopsis*, *Schizachne*, *Scolochloa*, and *Trisetum montanum*. Johnson (5) and Sprague (17) pointed out the similarity of *S. avenae* and

Stagonospora arenaria Sacc., the latter having been recorded on many grasses. Sprague maintains *Stagonospora arenaria* as a distinct species until more information is available. Shaw (13) tested species of *Agropyron*, *Anthoxanthum*, *Arrhenatherum*, *Dactylis*, *Elymus*, *Festuca*, *Hordeum*, *Secale*, *Stipa*, and *Triticum* with *S. avenae* isolated from *Avena sterilis* in Australia but no infection was obtained, although species of *Avena* were susceptible.

In this study no isolate which was later found to be pathogenic on oats was originally isolated from either wheat, barley, rye, or grasses, even from senescent material. It may be found later that the oat-infecting organism can infect or be carried over on other hosts besides oats, but there is no indication of this in the present study.

In order to check the point further several of the isolates were inoculated to green and faded leaves of *Agropyron intermedium*, *A. tenerum*, *Anthoxanthum odoratum*, *Calamagrostis epigeios*, *Elymus canadensis*, *E. virginicus*, *Glyceria striata*, *Hordeum jubatum*, *Poa compressa*, *P. pratensis*, and *Schizachne purpurea*. However, no infection was obtained. It would seem, therefore, that the oat-infecting organism is confined to oats. The question of *Stagonospora arenaria* is still unresolved, and the position remains as discussed by Sprague (17).

In 1947 Johnson (5) described *L. avenaria* f. sp. *triticea*, which could not infect oats but which was weakly parasitic on wheat. Article 34 of the International Code of Botanical Nomenclature (Lanjouw (8)) states: "The description of a subordinated taxon automatically creates a second subordinated taxon of the same rank which has as its nomenclatural type the type of the higher taxon." Also, Article 35 states: "If any infra-specific taxon which includes the nomenclatural type of the epithet of the next higher taxon is to be mentioned by a subdivisional name, that name must repeat the epithet of the higher taxon unaltered, but, contrary to Article 55, without citation of an author's name."

This is interpreted to mean, in the case under discussion, that with the erection of the special form *triticea*, the type becomes *L. avenaria* Weber f. sp. *avenaria* automatically (imperfect *S. avenae* f. sp. *avenae*).

In future, therefore, no collection should be designated *L. avenaria* f. sp. *avenaria* unless it is shown to be pathogenic on oats. Collections which are morphologically similar, but whose pathogenicity is unknown, can be designated *L. avenaria*.

Acknowledgments

Grateful acknowledgment is made to the Officer-in-Charge, Dr. T. Johnson, for the facilities provided at the Plant Pathology Laboratory, Science Service, Canada Department of Agriculture, Winnipeg; to Dr. D. Löve for identifying grass specimens; and to Mr. Walter Clark and Dr. R. C. McGinnis for taking the photographs. The writer also wishes to acknowledge financial assistance from Science Service during part of the research. It is a pleasure to acknowledge my gratitude to Dr. T. Johnson for his advice throughout this study. The study was carried out during the tenure of a Thomas Lawrance Pawlett Scholarship from the University of Sydney.

References

1. DRAYTON, F. L. A summary of the prevalence of plant diseases in the Dominion of Canada. Can. Dept. Agr. Bull. No. 71 (N.S.), (1926).
2. FRANDSEN, N. O. *Septoria*-Arten des Getreides und anderer Gräser in Dänemark. Medd. plantepat. afd. Kgl. Veterinaer- og Landbohejskole, København. No. 26. 1943.
3. HUFFMAN, M. D. Disease cycle of Septoria disease of oats. Phytopathology, **45**, 278–280 (1955).
4. JACOBSON, W. and WEBB, M. Nucleoproteins and cell division. Endeavour, **11**, 200–207 (1952).
5. JOHNSON, T. A form of *Leptosphaeria avenaria* on wheat in Canada. Can. J. Research, C, **25**, 259–270 (1947).
6. JOHNSON, T. Cultural variability in *Septoria avenae* Frank. Can. J. Botany, **30**, 318–330 (1952).
7. JONES, D. R. H. Variation in culture of *Fusarium culmorum*. Nature, **173**, 37–38 (1954).
8. LANJOUW, J. *et al.* International code of botanical nomenclature. Chronica Botanica Company, Waltham, Mass. 1952.
9. MEEHAN, F. and MURPHY, H. C. *Septoria avenae* on oats in Iowa. Phytopathology (Abstr.), **39**, 15 (1949).
10. MURRAY, R. G. E., GILLEN, D. H., and HEAVY, F. C. Cytological changes in *Escherichia coli* produced by infection with phase T2. J. Bacteriol. **59**, 603–615 (1950).
11. NITIMARGI, N. M. Studies on the genera *Cytosporina*, *Phomopsis* and *Diaporthe*. VII. Chemical factors influencing sporing characters. Ann. Botany (London), **49**, 19–40 (1935).
12. NOBLE, M. and MONTGOMERIE, I. G. Seed-borne oat diseases. Straw-breaking and brown foot-rot. Scot. Agr. **34**, 2–3 (1954).
13. SHAW, D. E. Studies on species of *Septoria*, *Stagonospora*, *Selenophoma* and related *Leptosphaeria* on the Gramineae in Australia. M.Sc. Agr. Thesis, University of Sydney, Sydney, Australia. 1953.
14. SHAW, D. E. Cytology of *Septoria* and *Selenophoma* spores. Proc. Linnean Soc. N. S. Wales, **78**, 122–130 (1953).
15. SHAW, D. E. Studies on *Leptosphaeria avenaria* f. sp. *triticea* on cereals and grasses. Can. J. Botany, **35**, 113–118 (1957).
16. SIMONS, M. D. and MURPHY, H. C. Kernel blight phase of Septoria black stem of oats. Plant Disease Reptr. **36**, 448–449 (1952).
17. SPRAGUE, R. Diseases of cereals and grasses in North America. The Ronald Press Company, New York, 1950.
18. SPRAGUE, R. Some leafspot fungi on Western Gramineae. VII. Mycologia, **46**, 76–88 (1954).
19. SPRAGUE, R. Some leafspot fungi on Western Gramineae. VIII. Mycologia, **47**, 249–262 (1955).
20. SPRAGUE, R. and FISCHER, G. W. Check list of the diseases of grasses and cereals in the Western United States and Alaska. Wash. Agr. Expt. Sta. Circ. 194 (1952).
21. STANTON, T. R. Septoria black stem of oats abundant in most of the North Central States. Plant Disease Reptr. **36**, 439 (1952).
22. TULASNE, R. and VENDRELEY, R. Demonstration of bacterial nuclei with ribonuclease. Nature, **160**, 225–226 (1947).
23. WEBER, G. F. Septoria disease of cereals. 1. Speckled blotch of oats caused by Leptosphaeria. Phytopathology, **12**, 449–470 (1922).

STUDIES ON LEPTOSPHAERIA AVENARIA f. sp. TRITICEA ON CEREALS AND GRASSES[1]

DOROTHY E. SHAW[2]

Abstract

Cultures were established from pycnidiospores assignable to *Septoria avenae* Frank collected on wheat, oats, barley, rye, and grasses in Canada. Those cultures that were nonpathogenic on oats produced, in most cases, perithecia and viable ascospores of *Leptosphaeria avenaria* Weber, as well as the asexual spores. Cultures were also established from asci and ascospores assignable to *L. avenaria* collected on wheat, oats, barley, rye, and grasses. Those that were nonpathogenic on oats produced, in most cases, pycnidiospores of *S. avenae* as well as the sexual spores. Many of the isolates produced slight to trace infection on the wheat variety Gaza and trace infection on barley. Oats, rye, and certain grasses were immune. It is considered that the isolates are a plurivorous group slightly pathogenic on wheat and found on the senescent parts of other cereals and grasses, and that they are best assigned to *L. avenaria* f. sp. *triticea*. No microspores were found in any of the cultures. Perithecia with viable ascospores formed in 123 cultures out of 164 established from single ascospores, some of the lines being maintained by single ascospore transfer up to five generations. This special form is therefore regarded as homothallic.

Introduction

Pycnidiospores assignable to *Septoria avenae* Frank and asci and ascospores assignable to *Leptosphaeria avenaria* Weber were isolated from wheat, oats, barley, rye, and grasses in Canada, in order to discover the cultural and pathogenic characteristics of the isolates and to determine whether they are homothallic or heterothallic. Isolates of *S. nodorum* from wheat from England and Canada were included in the study for comparative purposes. The methods used were the same as those reported elsewhere (5).

Cultural and Pathogenic Behavior

The isolates from oats that were vigorously pathogenic on oats in inoculation tests are dealt with elsewhere (5). All the other isolates were nonpathogenic on oats and were from spores from pycnidia or perithecia on wheat leaves or on senescent leaves, sheaths, or stems of the cereals and some grasses.

The details of the isolates, the spore forms produced in culture, and the pathogenicity tests are summarized in Table I.

The isolates shown in the table conformed in morphology to *L. avenaria* (imperfect stage *S. avenae*), but did not attack oats and therefore were not *L. avenaria* f. sp. *avenaria*. They also differed culturally from the oat form. The isolates were not vigorously pathogenic on wheat, and so could be easily distinguished from *S. nodorum*, as indeed they could be so distinguished on morphological grounds. Culturally they were also distinct from *S. nodorum* isolates. The isolates were, in most cases, either derived from macrospores,

[1]Manuscript received October 19, 1956.

[2]Thomas Lawrance Pawlett Scholar of the University of Sydney at the Science Service Laboratory, Winnipeg, Canada. Present address: Pathologist, Department of Agriculture, Stock and Fisheries, Port Moresby, Papua-New Guinea.

TABLE I

DETAILS OF COLLECTIONS, SUMMARY OF SPORE FORMS PRODUCED IN CULTURE, AND PATHOGENICITY TESTS WITH 44 ISOLATES

Cultures isolated from:	No. of collections represented	Original isolates	No. of collections forming spore forms in culture		Pathogenicity			
			Macro	Asco	Wheat	Barley	Rye	Oats
Wheat	11	Macro	4	9	6 + 5 tr	6 + 5 tr	—	—
Wheat	2	Entire asci	1	2	+	tr	—	—
Wheat	2	Asco	2	2	tr	—	—	—
Barley grain	1	Mycelium	—	1	+	—	—	—
Barley	2	Macro	—	1	1 + 1 tr	—	—	—
Barley	1	Entire asci	1	—	—	—	—	—
Rye	4	Macro	2	3	+	tr	—	—
Oats	5	Macro	5	5	3 + 2 tr	—	—	—
Oats	1	Entire asci	1	1	+	—	—	—
Oats	4	Asco	4	4	1 + 3 tr	—	—	—
Agropyron subsecundum	1	Entire asci Asco	1	1	+	tr	—	—
A. trachycaulum	1	Macro	—	1	+	—	—	—
A. trachycaulum	1	Entire asci	—	1	tr	—	—	—
Agropyron sp.	1	Macro	—	1	tr	—	—	—
Calamagrostis canadensis	1	Asco	1	1	tr	—	—	—
Elymus canadensis	2	Macro	2	2	1 + 1 tr	—	—	—
E. virginicus	1	Macro	—	1	+	—	—	—
Hordeum jubatum	1	Macro	—	—	—	—	—	—
Hordeum jubatum	1	Asco	—	1	tr	—	—	—
Poa pratensis	1	Entire asci Asco	—	1	+	—	—	—

NOTE: Collections came from seven provinces; macro = macropycnidiospore; asco = ascospore; + signifies that culture was weakly pathogenic; − signifies that culture was not pathogenic; tr = trace of infection.

or formed macrospores in culture, and were therefore distinct from *L. tritici* (Gar.) Pass., which, according to Müller (3), does not have an asexual spore form.

Of the 44 isolates, 23 of the 28 established from macrospores produced perithecia in culture, the one isolate established from surface-sterilized barley seed produced perithecia, and 14 of the 15 established from asci or ascospores produced perithecia. Therefore, of the 44 isolates, 38 produced perithecia and ascospores in culture which conformed to *L. avenaria*, but, being unable to infect oats, they were not *L. avenaria* f. sp. *avenaria*. Of the 15 cultures established from entire asci or ascospores, 11 produced pycnidia and macrospores in culture which conformed to *S. avenae*, but, being unable to infect oats, they were not assigned to *L. avenaria* f. sp. *avenaria*. Of the eight cultures which did not form perithecia, three did not produce pycnidia either, although small black initials were found on some media; three produced pycnidia, though very sparingly, and two were too young at the conclusion of the work to have produced any spores. However, the morphology of the spores present, the infection tests, and the cultural characteristics of these isolates were very similar to the others.

Twenty-six of the isolates were slightly pathogenic on the wheat variety Gaza and 16 caused a trace infection. It is considered that these isolates are best assigned to *L. avenaria* f. sp. *triticea*. Two of the isolates did not

produce even a trace infection on wheat, and while they are assigned to the species proper, it is quite possible that they are part of the population of *L. avenaria* f. sp. *triticea*. Nine of the isolates produced a trace infection on barley, but all were unable to infect rye or oats. The isolates from grasses were unable to infect the grasses from which they were isolated.

Isolate No. 168 from *Calamagrostis canadensis* was inoculated on green and fading leaves of *Agropyron intermedium*, *A. tenerum*, *Anthoxanthum odoratum*, *Elymus canadensis*, *E. virginicus*, *Festuca elatior*, *Hordeum jubatum*, and *Phleum pratense*. No infection was obtained on these species although a trace infection was recorded on the wheat variety Gaza.

The size of the ascospores and macrospores agreed well with those described by Johnson (2) for f. sp. *triticea*, the macrospores generally being a little smaller than those of the oat form, as shown in Table II for some of the isolates.

TABLE II

SIZES OF MACROSPORES OF *L. avenaria* f. sp. *avenaria* AND *L. avenaria* f. sp. *triticea* FROM VARIOUS HOSTS

Isolate	Host		Septations	Size of macropycnidiospores, μ
L. avenaria f. sp. *avenaria*	Oats	C*	3	33.5–46 × 2.5–3
" "	"	C	3(4)	31 –45 × 2.5–3
L. avenaria f. sp. *triticea*	Wheat	N*	3	32 –35 × 2.5–3
" "	Rye	N	3	29.5–39 × 2 –2.5
" "	Oats	N	3	28 –39 × 2.5
" "	*Agropyron trachycaulum*	N	3(2, 1)	28 –36.5 × 2.5
" "	*Agropyron* sp.	N	3	28 –39 × 2.5–3
" "	*Calamagrostis canadensis*	C	3(4)	31 –40.5 × (2)2.5(3)
" "	*Elymus canadensis*	N	3(1)	29.5–36.5 × 2.5–3
" "	*Elymus canadensis*	N	3	25 –39 × 2.5–3
" "	*Elymus virginicus*	C	3(1, 2)	26.5–33.5 × 2.5–3
" "	*Hordeum jubatum*	N	3	25 –36.5 × 2.5–3

*Spores from culture (C) or nature (N).

No microspores were found in any of the cultures on any medium, under various conditions of temperature and humidity.

The ease with which the form *triticea* forms perithecia in culture should be noted, also the fact that perithecia with mature ascospores were found in nature both before winter on mature plants and after winter on stubble. Shaw (4) noted that in Australia perithecia with mature spores of this form were found on mature wheat after winter and on stubble after summer. The ability of the form *triticea* to produce perithecia with ease in culture and under a wide range of conditions in the field differs from that of the oat form, whose sexual stage has been recorded only once in culture (7), and as far as is known, occurs only on overwintered oat stubble (1, 5).

As noted previously, the isolates from grasses were assigned to the form *triticea*, because of the immunity of oats and the grasses tested, and their weak pathogenicity on the wheat variety Gaza. It must not be overlooked that some of these isolates, however, might be vigorously pathogenic on other

grasses. Nor does it follow that all isolates whose asexual spores conform to *S. avenae* will have *L. avenaria* as the sexual stage. Conversely, isolates whose sexual stage conforms to *L. avenaria* might not have *S. avenae* as the asexual stage. Johnson (private communication) noted differences in length of macrospores from some grass hosts. These length differences might not have been due to environment.

Johnson (2) pointed out that the special form *triticea* has low parasitic vigor, even on wheat, and this was confirmed in the present study. From the information available, it appears that the form *triticea* is a plurivorous form weakly parasitic on wheat and found on the senescent parts of wheat, oats, barley, rye, and certain grasses.

The status of *Stagonospora arenaria*, as raised by Johnson (2) and Sprague (6), is still in abeyance. To the writer's knowledge no perfect stage has been reported for this species.

Homothallism of *L. avenaria* f. sp. *triticea*

In order to determine whether heterothallism or homothallism operates in this form, cultures were established as follows:

1. Single ascospores were isolated from perithecia on material from the field, or from perithecia produced in cultures which had originally been established from ascospores, asci, pycnidiospores, or mycelium, and the cultures were examined for perithecia and ascospores.

When evidence for homothallism was obtained, the isolates were subjected to the further test:

2. Lines were carried on by isolating single ascospores, obtaining perithecia, reisolating single ascospores, and so on, up to five generations.

The cultures were grown on sterile asparagus stems and citrus leaves, the most suitable media found so far for the production of perithecia.

TABLE III

NUMBER OF SINGLE ASCOSPORE CULTURES FORMING PERITHECIA

Acc.	Host	Originally isolated as:	No. of single ascospores isolated	No. of single ascospore cultures forming perithecia	No. of single ascospore cultures forming initials only
7	Wheat	Macro	18	9	6
8	"	"	6	6	
69	"	"	7	7	
73	"	"	8	5	2
94	"	Asco	6	5	1
154	"	"	3	3	
46	Rye	Macro	8	4	2
162	Oats	Asco	5	5	
172	"	"	8	8	
22	*Elymus canadensis*	Macro	12	7	4
168	*Calamagrostis canadensis*	Asco	6	6	
57	*Agropyron subsecundum*	Entire asci	12	8	1
65	*Poa pratensis*	" "	18	15	3
96	*Hordeum jubatum*	Asco	6	1	5
			124	89	24

(1) Production of Perithecia in Cultures Established from Single Ascospores

As shown in Table III, 124 single ascospores were isolated (by the method outlined by Shaw (5)) and perithecia with mature, pigmented ascospores formed in 89 of the cultures established from them. Twenty-four of the cultures which did not form perithecia with ascospores produced black bodies with no distinguishable contents. Eleven cultures were sterile.

The single ascospore cultures which did not produce perithecia were mated in all combinations on sterile citrus leaves, but no perithecia were obtained.

The above data strongly suggested that this organism is homothallic, and that those cultures established from single ascospores which did not produce perithecia were probably sterile only because of unsuitable media which either prevented perithecia from forming asci or spores, or prevented perithecial formation altogether.

(2) Lines Carried on by Single Ascospores

Shown in Table IV are selections of four lines, all maintained by transference of single ascospores. Perithecia with viable ascospores were obtained in each transfer up to the fifth generation. Of 40 single ascospore cultures established, 34 formed perithecia.

TABLE IV

PRODUCTION OF PERITHECIA IN LINES MAINTAINED BY SINGLE ASCOSPORES

Acc.	No. of ascospores isolated	No. of ascospore cultures forming perithecia	No. of generations represented
172	12	10	4
94	11	11	4
73	11	8	4
168	6	5	5
Total	40	34	

The total number of single ascospore cultures established in the two procedures (1 and 2) was 164, of which 123 formed fertile perithecia and 41 did not. It is not known that there is any significance in this perfect 3 : 1 ratio. As shown elsewhere (5) the ascospores are probably originally uninucleate, and not binucleate as in *Neurospora tetrasperma*, *Pleurage anserina*, and *Gelasinospora tetrasperma*, which have been termed "secondarily homothallic", each ascospore containing both compatibility factors.

It is to be noted that no difference in behavior was recorded for isolates either from different sources (wheat, rye, barley, oats, and grasses) or from different localities in several distinct regions.

It is concluded that *L. avenaria* f. sp. *triticea* is homothallic, mycelium from a single haploid ascospore having the ability to produce fertile perithecia. The final test, the demonstration that the eight single ascospores from one ascus can each produce perithecia with fertile ascospores, is yet to come.

Acknowledgments

Grateful acknowledgment is made to the Officer-in-Charge, Dr. T. Johnson, for the facilities provided at the Plant Pathology Laboratory, Science Service, Canada Department of Agriculture, Winnipeg, and for his advice throughout this study, which was carried out during the tenure of a Thomas Lawrance Pawlett Scholarship from the University of Sydney. The writer also wishes to acknowledge financial assistance from Science Service during part of the research.

References

1. HUFFMAN, M. D. Disease cycle of Septoria disease of oats. Phytopathology, **45**, 278–280 (1955).
2. JOHNSON, T. A form of *Leptosphaeria avenaria* on wheat in Canada. Can. J. Research, C, **25**, 259–270 (1947).
3. MÜLLER, E. Pilzliche Erreger der Getreideblattdürre. Phytopathol. Z. **19**, 403–416 (1952).
4. SHAW, D. E. Studies on species of *Septoria*, *Stagonospora*, *Selenophoma* and related *Leptosphaeria* on the Gramineae in Australia. M. Sc. Agr. Thesis, University of Sydney, Sydney, Australia. 1953.
5. SHAW, D. E. Studies on *Leptosphaeria avenaria* f. sp. *avenaria*. Can. J. Botany, **35**, 97–112 (1957).
6. SPRAGUE, R. Diseases of cereals and grasses in North America. The Ronald Press Co., New York. 1950.
7. WEBER, G. F. Septoria diseases of cereals. I. Speckled blotch of oats caused by *Leptosphaeria*. Phytopathology, **12**, 449–470 (1922).

STUDIES OF COPROPHILOUS ASCOMYCETES

V. SPHAERONAEMELLA FIMICOLA[1]

ROY F. CAIN AND LUELLA K. WERESUB

Abstract

An emended description of *Sphaeronaemella fimicola* Marchal refers this species to its proper taxonomic position among the Hypocreaceae, with both perfect and imperfect stages very similar to those of *S. helvellae* Karsten as described by Seeler. The species, found in nature on dung of various herbivorous animals (rabbit, deer, and cow), is culturable, growing more consistently on V-8 vegetable juice agar than on other media tried, most vigorously in association with other fungi like *Aspergillus repens* or *Microascus sordidus*, or with addition of filter-sterilized Czapek's broth in which *A. repens* had been grown. Fruiting in culture has been erratic; only early transfers from isolations made from field material yielded mature perithecia in pure culture, and then only on V-8 agar; later transfers fruited only in intimate contact with the mycelia of the other two fungi mentioned.

Introduction

The genus *Sphaeronaemella* was described by Karsten (7) with *Sphaeria helvellae* Karsten (6) as the type species. Because of the evanescence of the asci, the ascospores were mistaken for conidia, and the genus was placed in the Sphaeropsidales. The true nature of the spores was first fully described by Seeler (10), who transferred the genus to its proper place in the Nectriaceae and gave an emended description of the genus and species, including the asci, ascospores, and conidia. Details as to the method of formation of the conidia were not given.

S. helvellae has been reported from Finland, New Hampshire, New York, and Michigan, where it occurs on *Helvella infula*.

Several collections of a second species, *Sphaeronaemella fimicola* Marchal (8), have been made in Ontario and Quebec on the dung of rabbit, deer, and cow. Evanescent asci were observed in fresh specimens, and pure cultures were obtained by transferring ascospores from the spore-balls accumulated at the fimbriate ostioles of the long perithecial necks. A conidial stage was produced on all media tried but perithecia with mature ascospores occurred only on V-8 vegetable juice agar, and, after several transfers, only in association with certain other fungi.

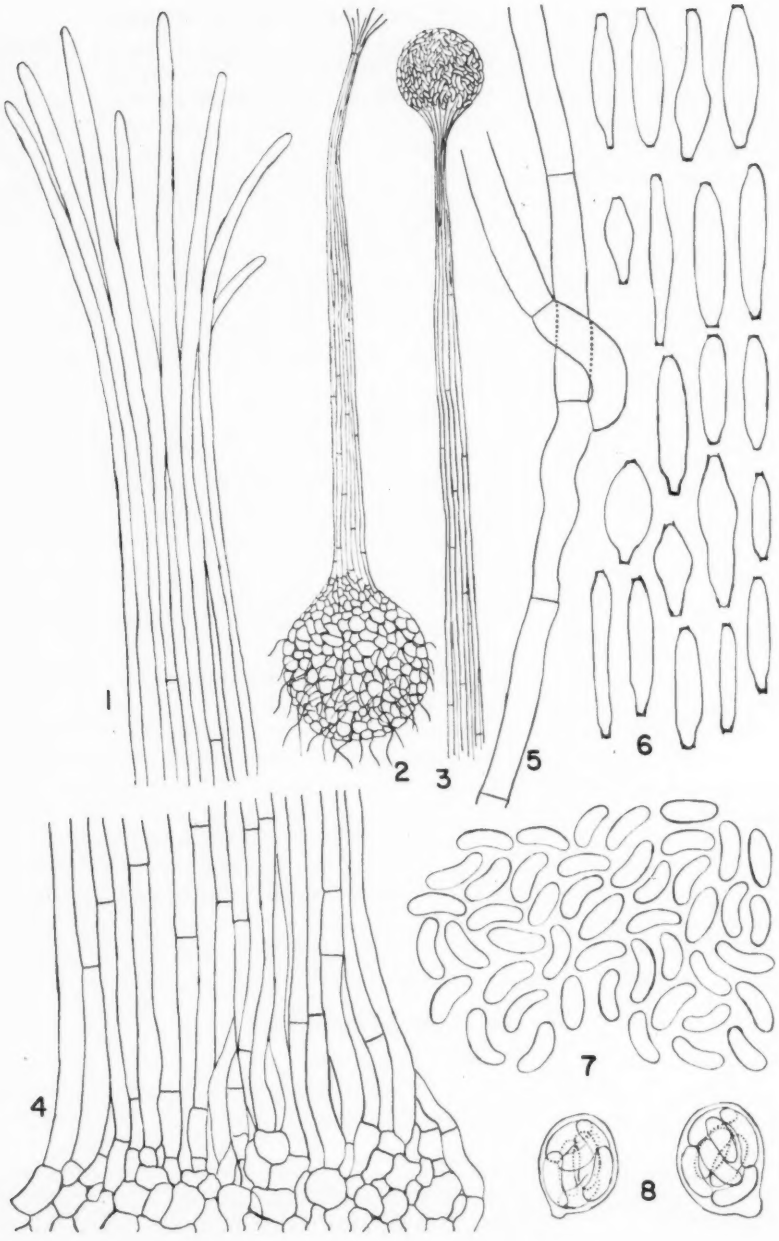
S. fimicola was described briefly by von Hoehnel (5), who surmised that it was an ascomycete although he did not see asci.

Emended Description

Sphaeronaemella fimicola Marchal (Figs. 1–30)

Perithecia scattered, globose, 150–300 μ diameter, light yellowish-brown, immersed to base of neck or partially emergent; peridium thin, membran-

[1]Manuscript received October 31, 1956.
Contribution from the Department of Botany, University of Toronto, Toronto, Canada. This study was carried out with assistance of a grant-in-aid of research from the National Research Council of Canada.


1
2
3
4
5
6
7
8

aceous, yellowish-amber, with rather large, conspicuous cells measuring up to 10(–12) μ diameter; neck long, straight, smooth, bare, light yellowish-brown, 500–700 μ long, 35–50 μ wide at base, 10–15 μ wide below apical fringe, consisting of parallel, remotely septate, laterally fused hyphae measuring 1.5–2.5 μ in width, terminated apically by an expanding fringe of obtusely pointed, separate, diverging hyphae, supporting a globose mass of ascospores. Asci subglobose, irregularly disposed, 12–15 $\times$ 10–12 μ, evanescent, 8-spored, expanding in crushed water mounts and quickly dissolving. Paraphyses lacking. Ascospores aggregated in a globose mass within the ascus, curved, almost allantoid, broadly rounded at the ends, smooth, hyaline, one-celled, 6–8 $\times$ 2.0–3.0 μ, passing uniseriately through the perithecial neck.

Colony on agar media white, thin, cottony with indefinite margin; hyphae hyaline, infrequently branched, mostly submerged, 3–4 μ diameter, remotely septate, only slightly constricted. Chlamydospores intercalary or lateral becoming thick-walled, globose, ellipsoid or irregular in shape, hyaline, 10–15 μ diameter. Phialides long, slender, slightly-tapered toward apex, hyaline, without flaring collarette, 36–52 μ in length, 4–5 μ in width near base and 1.6–2.0 μ in width at apex, usually at ends of undifferentiated aerial hyphae, less commonly laterally located just below septa, single when terminal and single or rarely in pairs when at a septum. Phialospores (10–)15–20 $\times$ 2.0–4.0 (mostly 2.5–3.5) μ, fusiform-oblong, broadest near the middle, slightly tapered toward each end, abruptly narrowed at distal end to a truncated apex, abruptly narrowed near lower end to a slender waist 2 μ in length, truncate at base, forming chains up to 400 μ in length, in water mounts rapidly separating, swelling to as much as 8 μ in width and germinating with production of a secondary conidium on a phialide-like projection or more rarely with a hypha.

Distribution

Specimens Examined

Ontario: *Lake Timagami*, Bear Island, Sept. 1927, on cow dung (TRTC 9224), Aug. 24, 1936, on deer dung (TRTC 31937), Sept. 9, 1933, on rabbit dung (TRTC 31938); Timagami Island, Aug. 10, 1931, on rabbit dung (TRTC 31939). *Peel Co.*, N. of Bolton, Oct. 8, 1955, on cow dung (obtained in pure culture) (TRTC 31350). All collected by R. F. C.

Quebec: *Laurentides Park*, Camp Mercier, Aug. 27, 1938, on deer dung, developed in laboratory, Toronto, Sept. 27, 1938, coll. R. F. C. (TRTC 31940). *Ungava*, Mt. Pyramid, 57° 30′ N. lat., Aug. 5, 1947, on wolf dung, coll. J. Rousseau (TRTC 31936).

FIGS. 1–27. *Sphaeronaemella fimicola*. All figures are $\times$1140 except Figs. 2 and 3 which are $\times$120.

FIG. 1. Apical region of perithecial neck showing hyphae fused to form a tube in lower part and diverging at apex. FIG. 2. Perithecium. FIG 3. Neck of perithecium with globule of ascospores at apex. FIG. 4. Basal region of perithecial neck. FIG. 5. Hypha. FIG. 6. Conidia, some of which are swollen after detachment. FIG. 7. Ascospores. FIG. 8. Two asci each with eight ascospores.

Production of Phialospores

(Figs. 9, 12)

The very long slender phialides form a loose layer in the aerial mycelium and are directed vertically or less commonly at a slight angle from the vertical. The hyaline phialospores are produced externally in succession at the undifferentiated apex of the phialide. They remain attached at the ends to form separate chains at least 300–400 μ long which droop in an arch and become somewhat entangled but never form a compact layer.

Germination of Phialospores

(Figs. 10, 11)

The hyaline phialospores swell soon after maturation and germinate promptly thereafter. Germination consists of the putting out of a slender projection, generally near one end, sometimes from the side, rarely from the apex. Infrequently, two such projections arise from a single phialospore. The projection usually extends out to a length of about 15 μ, curves, and tapers gracefully from the broadest part near the base to a slender apex measuring about 1 μ in diameter where it swells out to form a conidium somewhat smaller and more slender than the phialospore. Rarely a second conidium is formed below the apical one indicating that the projection from the germinating phialospore not only looks like a phialide but actually is one. This method of germination resembles, superficially, the germination by repetition of basidiospores in the Tremellaceae and other related families.

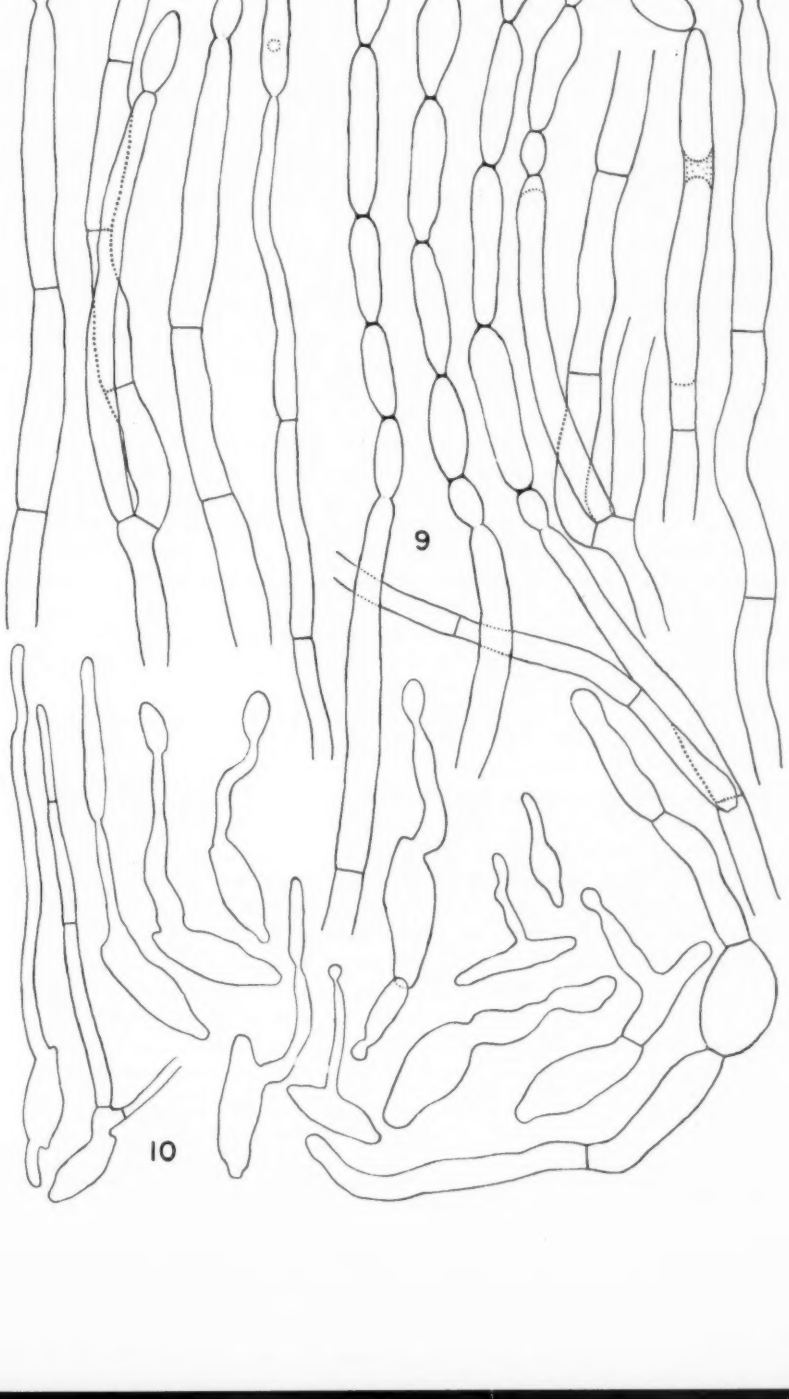
Growth in Culture

Fresh material fruiting on cow dung was brought into the laboratory on Oct. 8, 1955, and ascospores were seeded onto a modified Leonian's agar[2] and onto oatmeal agar[3]. On both media, the cultures grew slowly and produced a scanty white mycelium with some aerial growth which was mainly a loose layer of phialides with chains of phialospores. The agar slants were eventually covered in all tubes, but no perithecia appeared.

After about 3 weeks, transfers were made from several of the tubes to V-8 agar (9).[4] A more luxuriant growth followed, with a central portion of fairly dense cottony white mycelium, phialides, and phialospores, and an indefinite sparsely covered margin. After several weeks, one tube yielded a few perithecia with mature asci and ascospores. Subsequent transfers from this tube to each of the three types of media mentioned above made very

[2]Leonian's plus 0.1% yeast extract.
[3]Basal medium: ammonium nitrate 3.0 g., potassium dihydrogen phosphate 1.0 g., magnesium sulphate 0.6 g., potassium chloride 0.5 g., agar 20 g., tap water 1000 ml. Plus yeast extract 1 g.; oatmeal flakes.
[4]20% V-8 vegetable juice, 2% agar, 3% calcium carbonate.

FIG. 9. Ten phialides showing arrangement on the aerial hyphae or phialophores. Each has an attached phialospore in various stages of development or a chain of phialospores. FIG. 10. Phialospores germinating either with hyphae or secondary phialides and secondary phialospores.


9
10

little growth. The colonies extended only a few millimeters, and there was practically no aerial mycelium.

A similar lack of success attended transfers to plates and tubes of various media. On YpSs (4) and Sach's agar (11), growth was slow, mainly submerged, and quickly arrested. On horse dung plus basal-yeast agar,[3] a promising beginning of comparatively abundant superficial growth and sporulation led to little further development. Cultures of V-8 agar in plates lined with filter paper were sometimes encouragingly widespread, occasionally even fairly dense, but without production of perithecia or apparently even of ascogonia.

One of the V-8 Petri dish cultures became contaminated within 2 weeks after inoculation by a colony of *Aspergillus repens*. Thereafter, growth of the *Sphaeronaemella* intensified in the direction of the contaminant, while that of the latter was restricted. By the time the *Sphaeronaemella* was about 6 weeks old, that part of its colony which had approached and to some extent overgrown the *Aspergillus* was producing numerous perithecia with mature ascospores.

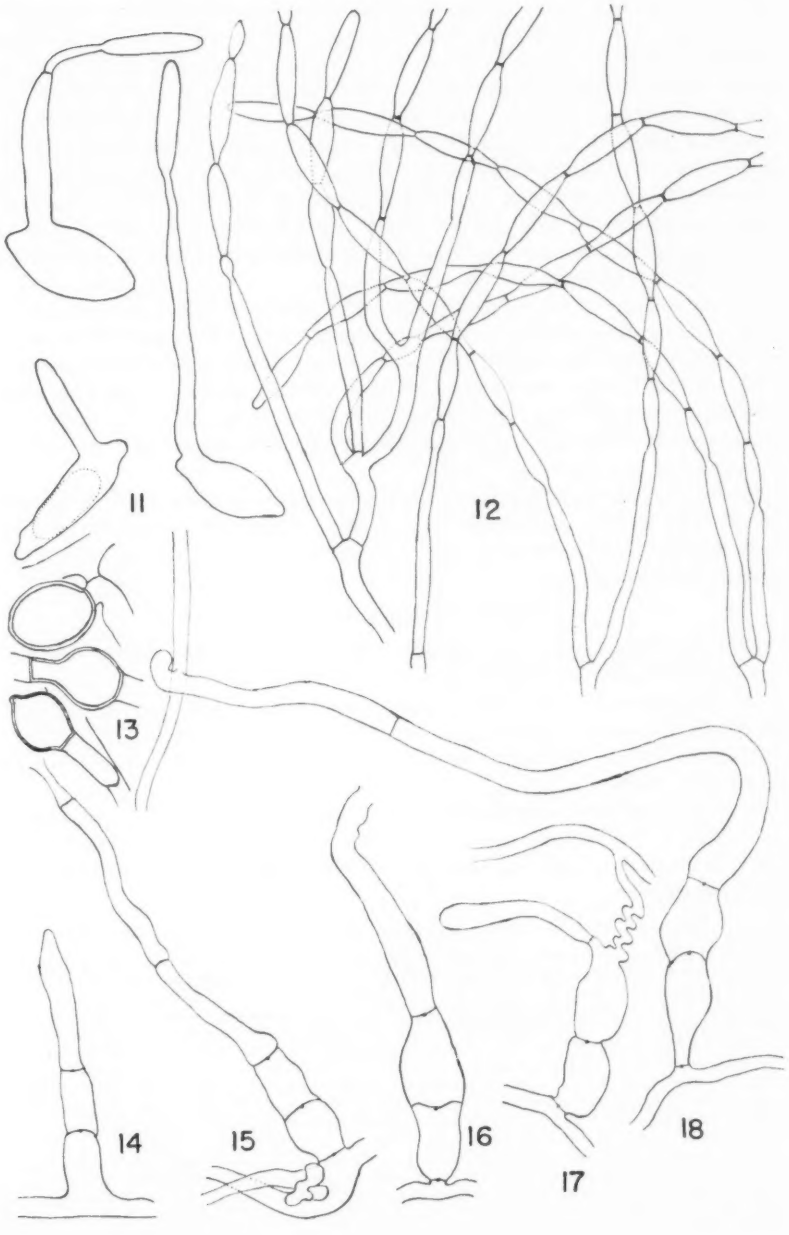
This led to the inoculation of plates of V-8 agar with *Sphaeronaemella fimicola* plus, at a distance of about 2 cm., *Aspergillus repens*, *A. ustus*, *A. niger*, *A. fumigatus*, or *A. clavatus*. The latter two quickly overgrew the *Sphaeronaemella*; *A. niger* at first somewhat stimulated and then advanced rapidly over it; *A. ustus* was itself at first restrained from spreading over the sphere of influence of the *Sphaeronaemella* whose growth it has apparently invigorated, but eventually extended itself over the entire plate. On the other hand, when plated with *A. repens*, the *Sphaeronaemella* definitely restricted growth of the other fungus, grew more or less luxuriantly, and in one case produced perithecia within 2 weeks.

Transfers from areas where the *Sphaeronaemella* and *A. repens* mycelia were intermingled yielded, on V-8, a rich cottony growth of the *Sphaeronaemella* type with no outward sign of the *Aspergillus*, and, in one case, proceeded to the point of fruiting. Buston and Khan (1) found that the time of inoculation and distance apart of inocula were significant factors influencing the numbers of perithecia produced by *Chaetomium globosum* in association with *Aspergillus fumigatus* and that the *Chaetomium* could inhibit the growth of the *Aspergillus*.

In an attempt to secure mature perithecia in pure culture, 11-day-old plate cultures of the *Sphaeronaemella* were flooded with 1.5 cc. Zeitz-filtered Czapek's broth (20% sucrose) in which *A. repens* had been growing for 11 days; and 5-day-old tube cultures received 1 cc. broth from 24-day-old growth. In the former case, there were some slight indications of a stimulatory effect on

---

FIG. 11. Germinating phialospores. FIG. 12. Phialides with interlocking chains of phialospores. FIG. 13. Chlamydospores, terminal on branchlets, more or less sessile or intercalary. FIG. 14. Young ascogonium with apical cell forming the trichogyne. FIG. 15. Ascogonium with two swollen cells and three-celled trichogyne which is broken at the apex where it has been torn away at the point of fusion with a hypha. FIG. 16. Ascogonium with two swollen cells and one-celled trichogyne torn at the point of fusion. FIG 17. Ascogonium with branched trichogyne, one branch fused with a hypha. FIG. 18. Ascogonium with two-celled trichogyne fused with a hypha.


11
12
13
14
15
16
17
18

Sphaeronaemella growth. The results of the latter are to be seen in Fig. 29: comparison of I and IV reveals the greater nutritive value of V-8 agar over modified Leonian's, since in I, on Leonian's, growth is concentrated at the point of inoculation, whereas in IV, on V-8, mycelium and sporophores cover the entire surface of the agar, though sparsely. II shows that filtered Czapek's broth in itself provides little, if any, stimulus to growth, while III and V demonstrate the enhanced growth that is due to the addition of filter-sterilized broth in which *A. repens* has been growing for 24 days. Thus we have conclusive evidence that *A. repens* either excretes into the medium or converts medial components into some filterable factor which is required by *S. fimicola* for optimum growth.

In summary, with regard to the vegetative growth of this fungus, it may be said that, of the media used in these studies, V-8 agar proved to be most satisfactory, but was insufficient in itself to provide subcultured material with all the factors required for most vigorous growth. The additional factor(s) necessary can be supplied by other fungi (e.g. *A. repens*) if introduced at the proper stage of development of the *Sphaeronaemella* and at the desirable concentration.

Under all conditions, vegetative development was consistently as described, except that the more vigorous cultures showed increased branching of the hyphae and the older cultures a higher frequency of thicker-walled chlamydospores of various shapes.

Perithecial Production in Culture

Following the isolation of ascospores from fruiting field material, perithecial production in artificial culture occurred on V-8 agar:

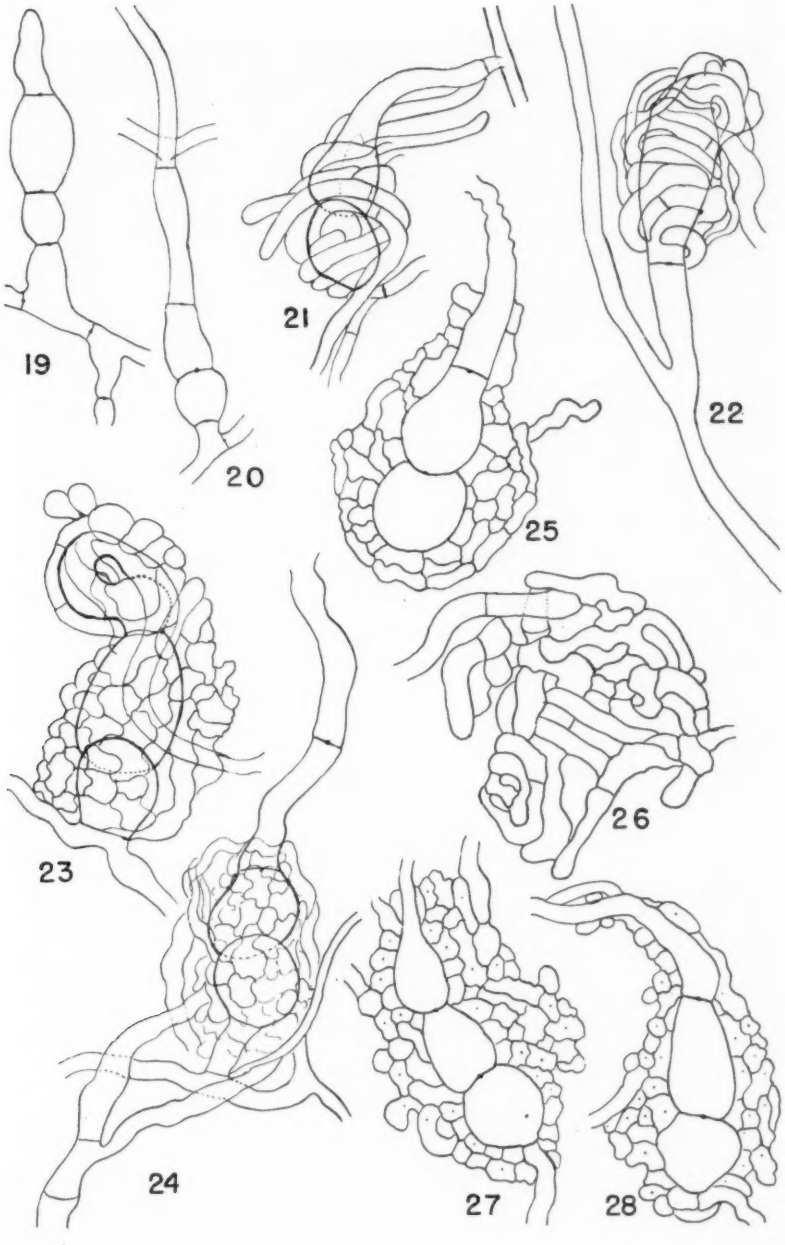
(*a*) after about 6 weeks from a transfer of 3 weeks' growth of the original isolate; and in later transfers:

(*b*) after about 6 weeks in association with a 4-week-old contaminant of *Aspergillus repens;*

(*c*) after 2 weeks in association with an *A. repens* colony of identical age;

(*d*) after a few weeks in association with a colony of *Microascus sordidus* which was already growing actively at the time of introduction of the *Sphaeronaemella*;

FIG. 19. Young ascogonium with two swollen cells and trichogyne. FIG. 20. Ascogonium showing lateral fusion of trichogyne and a hypha. FIG. 21. Ascogonium in more advanced stage showing two large swollen cells, trichogyne fused with a hypha and perithecial peridium beginning its development from adjoining and basal hyphae that twine around the ascogonium. FIG. 22. Young ascogonium as a side branch on a hypha being enclosed by peridium-forming hyphae. FIG. 23. Ascogonium with two large swollen cells and a trichogyne which is twisted in two regions and curved back over the partly hyphal and partly pseudoparenchymatous peridium which encloses the ascogonium and base of the trichogyne. FIG. 24. Ascogonium with trichogyne torn at apex and peridium developed in part from adjoining hyphae. FIG. 25. Optical section of peridium-covered ascogonium from crushed mount, showing two large swollen cells and trichogyne torn at apex (only median portion of peridium has been drawn). FIG. 26. Surface view of peridium covering ascogonium shown in previous figure. FIG. 27. Ascogonium with trichogyne swollen at base; from sectioned material. FIG. 28. Ascogonium with two swollen cells and trichogyne enclosed in pseudoparenchymatous peridium, the cells of which are apparently uninucleate; from sectioned material.


19
20
21
22
23
24
25
26
27
28

(*e*) after a few weeks in association with both *A. repens* and *M. sordidus*, inoculation of the *Sphaeronaemella–Aspergillus* combination and of the *Microascus* having been made simultaneously;

(*f*) after a few weeks of growth from an inoculum composed of mingled *Sphaeronaemella* and *Aspergillus* mycelium.

On one plate of V-8 agar on which *A. repens* had been growing for 2 weeks, the introduction of *Sphaeronaemella* inoculum from a fruiting culture resulted in production of ascogonia and perithecial initials within a few weeks; but these developed no further.

Since Doguet (3) had found *Cytisus laburnum* so effective in stimulating fruiting of *Melanospora*, seeds of this legume were surface-sterilized with chlorine solution, split, and placed on V-8 agar along with *Sphaeronaemella* inoculum. No perithecia resulted.

These observations were made in the course of an effort to keep *Sphaeronaemella fimicola* fruiting in order to investigate its morphological development. Therefore, since no systematic attempt was made to trace the physiological factors involved in perithecial production, few unequivocal conclusions can be drawn.

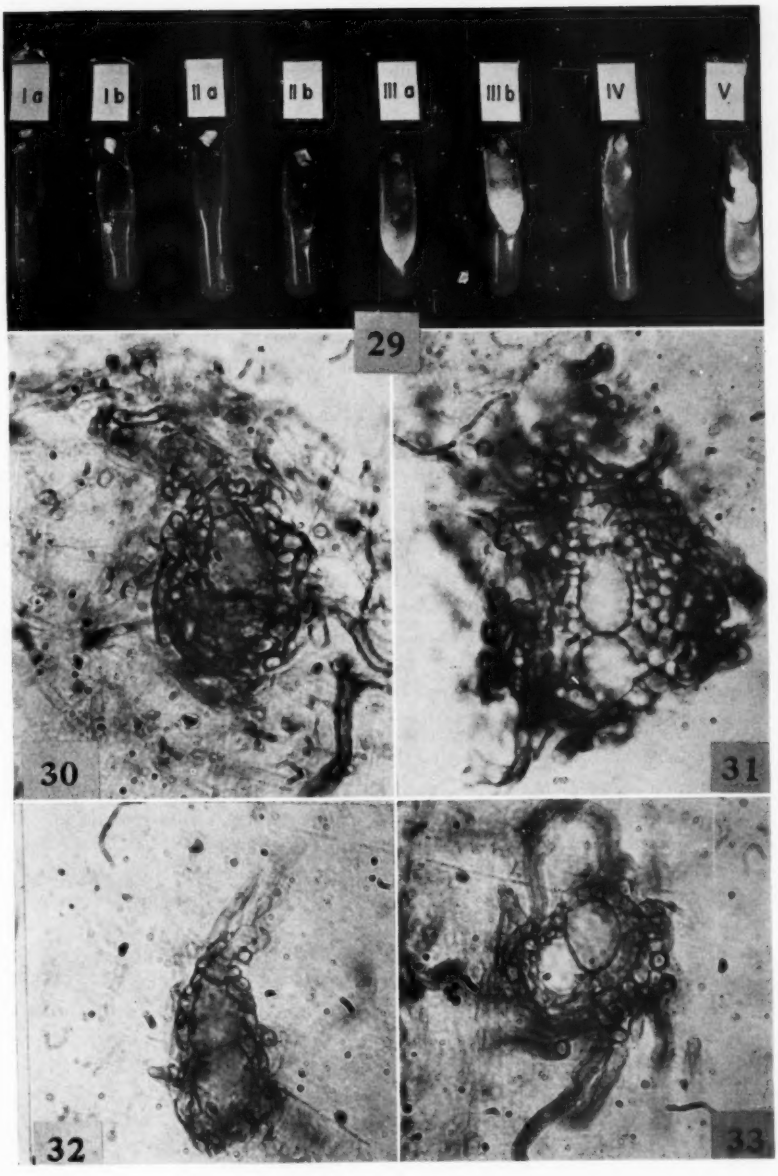
The explanation of the observed phenomena may lie in the likelihood that the fungus develops in its natural habitat in close association with other fungi which provide it with the necessary stimulants to fruiting. Ascospores from field material apparently retain sufficient store of the factor(s) to produce mycelium capable of developing, under proper conditions, to the point of maturation of perithecia; but further subculturing results in exhaustion of the stimulants, and fruiting under the conditions provided in our culturing fails to accumulate enough for carryover in transfer.

Since growth of the *Sphaeronaemella* was enhanced by 24-day-old *Aspergillus* medium administered to 5-day-old cultures, while fruiting did not result, it appears that either a different factor affects fruiting or the same factor must appear at a different stage in *Sphaeronaemella* development or at a different concentration. Two other observations may be significant in this regard: the abortion of ascogonia when the donor colony was older and at some distance from the recipient mycelium; and the synchronized fruiting that normally occurs, with all perithecia in a colony being at approximately the same stage of development at the same time. It may be that the decisive circumstance is the continuous production of the factor(s) over a specific period in the

---

FIG. 29. Growth of *Sphaeronaemella fimicola* as affected by broth in which *Aspergillus repens* has been cultured.
I–III: modified Leonian's agar; *a* and *b*: replicates.
IV and V: V-8 agar.
I–V: inoculated with *S. fimicola* on same date, photographed after 7 weeks.
I and IV: control: *Sphaeronaemella* alone.
II: control: after 5 days, introduction of 1 cc. Zeitz-filtered Czapek's 20% sucrose broth.
III and V: after 5 days, introduction of 1 cc. Zeitz-filtered Czapek's broth from 24-day-old culture of *A. repens*.

FIGS. 30–33. Sectioned material showing ascogonia with two large cells and trichogyne surrounded by the peridium. In the culture from which this material was taken the ascocarps did not develop beyond this stage.



Cain and Weresub—Can. J. Botany

development of the *Sphaeronaemella*. On the other hand, since perithecial production occurred only where the mycelia of the recipient and donor were intermingled, perhaps a more or less intimate contact between the two is necessary for passage of the factor(s) from one to the other. If either is the case, *Sphaeronaemella fimicola* may be considered a true parasite in nature, since it would be dependent at least for part of its life cycle on association with another living organism.

Perithecial Development

The ascogonia develop as short side branches (Figs. 14, 19) from hyphae which are deeply embedded in a dense, compact mat of white mycelium. Consequently there is considerable difficulty in obtaining clear mounts from dissected material. However, it was possible to make the following observations: The branch becomes two to four septate (Figs. 14–19) and the two lower cells enlarge to a globose shape (Figs. 20–28, 30–33). The upper part is a long, cylindrical, tortuous trichogyne (Figs. 15–28). In a few instances only one enlarged cell could be detected (Fig. 22) and sometimes the basal part of the trichogyne is swollen (Fig. 27). Hyphae grow out from the base of the ascogonium and twine around it to form the peridium of the ascocarp (Figs. 21, 22). Adjoining hyphae also become involved (Figs. 22, 24) and the enlarged cells as well as the basal part of the trichogyne are soon enclosed in a pseudoparenchymatous layer of irregular cells (Figs. 23–28, 30–33). The trichogyne fuses with a separate hypha either at the end (Fig. 21) or along the side (Figs. 18, 20). In teased mounts, the end usually becomes torn away at the point of fusion leaving an open end (Figs. 15, 16, 23, 24, 25). With the increased thickening of the ascocarp peridium, sectioning would have been necessary for observation of further stages of development. Unfortunately suitable material for such study was not available.

Relationships

Sphaeronaemella fimicola appears to be closely related to *S. helvellae* and is distinguished mainly by the narrower ascospores and the habitat. With regard to the distinction in habitat: *S. helvellae* has been found only on *Helvella infula* on which it is apparently parasitic, *S. fimicola* only on dung. However, the latter may well be parasitic also, since in artificial culture, with one exception, it has produced no perithecia on heat sterilized dung or other media except in association with other fungi. Species of Hypocreaceae are known to occur on a wide variety of substrates under natural conditions, but many of these are probably actually dependent on the active mycelium of other fungi inhabiting these areas.

Seeler (10) has suggested that *Sphaeronaemella* may be closely related to *Melanospora*, but it appears that their similarities are superficial rather than phylogenetically fundamental. An excellent account of the latter genus is given by Doguet (3), in which he points out that *Melanospora* ascospores have a thickened and colored wall with a circular germ pore at each end. The ascospores of *Sphaeronaemella* are not so characterized. All of the species of

Melanospora that have been investigated in culture produce phialides and phialospores, but these are quite different from the corresponding structures developed in cultures of *Sphaeronaemella*. In the former genus the phialides are much shorter, with a broader base and more flaring collarette, and are usually formed laterally and scattered on the hyphae. The ovate phialospores accumulate in a ball at the apex of the phialide. In *Sphaeronaemella*, both phialides and phialospores are so much more highly evolved that the two genera cannot be considered very closely related. The bright color of the perithecia, their long necks, and their fimbriate ostioles are the chief characteristics common to both genera. The color and structure of the perithecial peridium probably indicates that the two genera are to be included in the Hypocreaceae. However, since the type of neck is an adaptation to ascospore dispersal by means of invertebrate animals and has almost certainly evolved in several lines, it cannot be taken by itself as proof of relationship. Therefore, in view of the differences in less adaptive and hence probably more fundamental characters, *Sphaeronaemella* and *Melanospora* appear to have had a distinctly separate origin.

Sphaeronaemella is closer to, but not congeneric with, *Ceratocystis*, being distinct in structure of perithecium, imperfect stage, and ascospore type. The latter genus itself, as it stands today, is a composite one containing species which probably belong to more than one line of evolution. Another related genus is *Microascus* whose perithecium, producing honey-yellow ascospores, lacks an ostiolar fringe and whose imperfect stages, when present, belong to *Scopulariopsis* and *Sporocybe*. The genus *Petriella*, a segregate from *Microascus* by Curzi (2) for species with scattered hairs on the perithecial base and neck, is not distinct and the name should be reduced to synonymy.

Species of *Melanospora*, *Sphaeronaemella*, *Microascus*, and *Ceratocystis* all have short evanescent asci which have evolved in adaptation to a specialized means of ascospore dispersal. It is likely that they have all had their origin from taxa more typical of the Hypocreaceae but probably not in a single evolutionary line.

Note added in proof.—The ascigerous nature of the fructification was previously reported by Pease (Mycologia, **40** (1), 114–124 (1948)) in an apparently similar fungus referred by her to *S. fimicola*. It was isolated from decaying cucurbit fruits and differs in several other respects from this species. Comparison of cultures under identical conditions would be necessary to determine whether her fungus belongs in the same species with the coprophilous one. *Fusidium parasiticum* Westendorp, as described by Backus and Stowell (Mycologia, **45**, 836–847 (1953)), may also be a related species.

References

1. Buston, H. W. and Khan, A. H. The influence of certain micro-organisms on the formation of perithecia by *Chaetomium globosum*. J. Gen. Microbiol. **14**(3) : 655–660 (1956).
2. Curzi, M. *Petriella*, nuovo genere di pirenomycete. Boll. regia staz. patol. vegetale, **10**(4), 380–422 (1930).

3. DOGUET, G. Le genre *Melanospora*, biologie, morphologie, développement, systématique. Botaniste, **39**, 1–313 (1955).
4. EMERSON, R. and WILSON, C. M. Interspecific hybrids and the cytogenetics and cytotaxonomy of *Eurallomyces*. Mycologia, **46**, 393–434 (1954).
5. HOEHNEL, F. VON. Fungi Imperfecti. Beitrage zur Kenntnis derselben. 58. Ueber die Gattung *Sphaeronaemella* Karsten. Hedwigia, **60**, 151–155 (1918).
6. KARSTEN, P. A. Fungi Fenniae exs. 674. 1867.
7. KARSTEN, P. A. Fragmenta mycologica. VIII. Hedwigia, **23**, 17 (1884).
8. MARCHAL, E. Champignons coprophiles. Bull. soc. roy. botan. Belg. **33**, 12 (1894).
9. MILLER, P. M. V-8 juice agar as a general purpose medium for fungi and bacteria. Phytopathology, **45**, 461–462 (1955).
10. SEELER, E. V. Several fungicolous fungi. Farlowia, **1**, 119–133 (1943).
11. SHOEMAKER, R. A. Biology, cytology, and taxonomy of *Cochliobolus sativus*. Can. J. Botany **33**, 562–576 (1955).

Notes to Contributors

Manuscripts

(i) ***General***

Manuscripts, in English or French, should be typewritten, double spaced, on paper 8½ × 11 in. **The original and one copy are to be submitted.** Tables and captions for the figures should be placed at the end of the manuscript. Every sheet of the manuscript should be numbered.

Style, arrangement, spelling, and abbreviations should conform to the usage of this journal. Names of all simple compounds, rather than their formulas, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed.

Manuscripts and illustrations should be carefully checked before they are submitted. Authors will be charged for unnecessary deviations from the usual format and for changes made in the proof that are considered excessive or unnecessary.

(ii) ***Abstract***

An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

(iii) ***References***

References should be listed **alphabetically by authors' names,** numbered, and typed after the text. The form of the citations should be that used in this journal; in references to papers in periodicals, titles should be given and inclusive page numbers are required. The names of periodicals should be abbreviated in the form given in the most recent *List of Periodicals Abstracted by Chemical Abstracts.* All citations should be checked with the original articles, and each one referred to in the text by the key number.

(iv) ***Tables***

Tables should be numbered in roman numerals and each table referred to in the text. Titles should always be given but should be brief; column headings should be brief and descriptive matter in the tables confined to a minimum. Vertical rules should be used only when they are essential. Numerous small tables should be avoided.

Illustrations

(i) ***General***

All figures (including each figure of the plates) should be numbered consecutively from 1 up, in arabic numerals, and each figure should be referred to in the text. The author's name, title of the paper, and figure number should be written in the lower left-hand corner of the sheets on which the illustrations appear. Captions should not be written on the illustrations (see Manuscripts (i)).

(ii) ***Line drawings***

Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in blue only; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in green, yellow, or red should not be used unless it is desired to have all the co-ordinate lines show. All lines should be of sufficient thickness to reproduce well. Decimal points, periods, and stippled dots should be solid black circles large enough to be reduced if necessary. Letters and numerals should be neatly made, preferably with a stencil **(do NOT use typewriting)**, and be of such size that the smallest lettering will be not less than 1 mm. high when reproduced in a cut 3 in. wide.

Many drawings are made too large; originals should not be more than 2 or 3 times the size of the desired reproduction. In large drawings or groups of drawings the ratio of height to width should conform to that of a journal page but the height should be adjusted to make allowance for the caption.

The original drawings and one set of clear copies (e.g. small photographs) are to be submitted.

(iii) ***Photographs***

Prints should be made on glossy paper, with strong contrasts. They should be trimmed so that essential features only are shown and mounted carefully, with rubber cement, on white cardboard with no space or only a **very** small space (less than 1 mm.) between them. In mounting, full use of the space available should be made (to reduce the number of cuts required) and the ratio of height to width should correspond to that of a journal page (4¾ × 7½ in.); however, allowance must be made for the captions. Photographs or groups of photographs should not be more than 2 or 3 times the size of the desired reproduction.

Photographs are to be submitted in duplicate; if they are to be reproduced in groups one set should be mounted, the duplicate set unmounted.

Reprints

A total of 50 reprints of each paper, without covers, are supplied free. Additional reprints, with or without covers, may be purchased.

Charges for reprints are based on the number of printed pages, which may be calculated approximately by multiplying by 0.6 the number of manuscript pages (double-spaced typewritten sheets, 8½ × 11 in.) and including the space occupied by illustrations. An additional charge is made for illustrations that appear as coated inserts. The cost per page is given on the reprint requisition which accompanies the galley.

Any reprints required in addition to those requested on the author's reprint requisition form must be ordered officially as soon as the paper has been accepted for publication.

Contents

	Page
The Biosynthesis of Radioactive Arabinose, Xylose, and Galactose Using Detached Wheat Seedlings—*Lillian M. Cowie and G. Krotkov*	1
The Constitution of Sugar Beet Seedlings Associated with Chemically Induced Improvement in Their Low Temperature Resistance—*Sherwood R. Miller and William G. Corns*	5
Soluble Carbohydrates of Wheat Stems and Leaves—*L. E. Lopatecki, E. L. Longair, and C. W. Farstad*	9
Developmental Studies of *Elodea canadensis* Michx. I. Morphological Development at the Shoot Apex—*Hugh M. Dale*	13
On the Extraction of Enzymes from Succulent Plants—*C. H. Coles and E. R. Waygood*	25
A Technique for the Sterilization and Inoculation of Wood Cut from Living Trees—*J. T. Basham*	31
Sedimentary Chlorophyll Degradation Products in Surface Muds from Connecticut Lakes—*J. R. Vallentyne and Dennis F. Craston*	35
Phomopsis Black Rot of Cucurbits—*Colin D. McKeen*	43
Developmental Studies of *Elodea canadensis* Michx. II. Experimental Studies on Morphological Effects of Darkness—*Hugh M. Dale*	51
A Comparative Study of Fungitoxicity and Phytotoxicity in an Homologous Series of N-*n*-alkylethylenethioureas—*R. G. Ross and R. A. Ludwig*	65
Studies on *Leptosphaeria avenaria* f. sp. *avenaria*—*Dorothy E. Shaw*	97
Studies on *Leptosphaeria avenaria* f. sp. *triticea* on Cereals and Grasses—*Dorothy E. Shaw*	113
Studies of Coprophilous Ascomycetes. V. *Sphaeronaemella fimicola*—*Roy F. Cain and Luella K. Weresub*	119

Wiki